PARASITOLOGY

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PARASITOLOGY

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VOLUME 34, No. 1 MAY 1942

CHEMOTHERAPY AND AVIAN MALARIA

BY ANN BISHOP, Sc.D.[1]

From the Molteno Institute, University of Cambridge

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1. INTRODUCTION

A. *Malaria as a world problem*

MALARIA is endemic over a wide area of the earth's surface. Although popularly associated with tropical or subtropical countries, it occurs as far north as the south of Sweden and Lake Ladoga in Russia and as far south as Bechuanaland, Swaziland and Natal in South Africa and the Argentine in South America. Though most frequent in low-lying districts it may be found at altitudes as great as 6000–9000 ft.

[1] The author is in receipt of a grant from the Medical Research Council, London.

One-third of the population of the world is said to be infected with malaria, but it is impossible to estimate the total yearly incidence with any degree of accuracy, as the disease is prevalent in undeveloped territories amongst primitive people as well as in highly developed countries. Some idea of its morbidity can be obtained from India where amongst a population of 348,000,000 it was estimated (League of Nations, 1932) that in 1929 100,000,000 cases of malaria occurred. James (1920) attributes an annual death-rate from malaria in India of 5 per 1000 of the population, i.e. 1,130,000 deaths, whilst according to Sinton (1935) at least one million deaths a year are due to endemic malaria, the number increasing to one and a quarter or to one and a half millions in years of severe regional epidemics. The malaria mortality rate is very difficult to assess, as many infected persons die from intercurrent diseases to which the lowering of their resistance by malaria has predisposed them. By its combined direct and indirect action Sinton considers that malaria is probably responsible for two million deaths in India annually. This death-rate is sufficiently enormous to impress even a generation inured to the casualties of modern warfare.

Apart from the annual loss of human life due directly or indirectly to endemic malaria there occur from time to time great epidemics of the disease. Such epidemics are frequent in India. The mortality at Amritsar in the malarial epidemic in the Punjab in 1908 was greater than in the epidemics of plague and cholera (League of Nations, 1930). In 1922–3 a pandemic of malaria occurred in Russia amongst a population whose resistance to the disease was already reduced by war, revolution, typhus, drought and famine. It is estimated that in the whole of Russia more than 12 million cases occurred (Gill, 1938). In many districts there was no machinery for the collection of statistics, but Mackenzie (1923) estimated that, in areas in which subtertian malaria formerly was unknown and benign tertian malaria rare, up to 90 % of the population was affected. In Turkestan the morbidity was enormous, and at Baku, where 27,000 cases were registered, the mortality amongst children was as great as 40 % (Tarassévitch, 1923). The disease was spread by troop movements, by the migrations of people to and from endemic areas in search of food, and later, as the pandemic reached terrifying dimensions, by fugitives from heavily infected districts. Antimalarial measures had broken down and quinine was almost completely lacking. Large tracts of land fell out of cultivation owing to the debility of the survivors, thus increasing the famine and lowering still further the resistance of the people to the infection. It is obvious that under such tragic conditions any accurate estimate of the number of deaths due specifically to malaria would be impossible.

More recently, a serious epidemic of malaria occurred in Ceylon. It started (Briercliffe & Dalrymple-Champneys, 1935*a*, 1935*b*) towards the end of 1934 and was followed by a secondary wave beginning in April 1935. In 1934 the south-west monsoon partly failed, causing great drought and a reduction in the flow of the rivers. The pools thus formed provided ideal breeding places for

Anopheles culicifacies, the only species of mosquito which has been proved to transmit malaria in Ceylon. A quarter of the whole surface of the island was affected by the epidemic. In the 6 months between October 1934 and April 1935 nearly one and a half millions of people contracted malaria, and between November 1934 and April 1935, 59,144 deaths occurred in the epidemic area in excess of the average number for the similar period during the four previous years. Some of these deaths could be ascribed directly to malaria, but the majority were due to intercurrent diseases such as dysentery, severe diarrhoea, and pneumonia, and only indirectly to malaria.

Malaria has played an important part in many military campaigns. Doubt may be expressed (Swellengrebel & de Buck, 1938) whether the disastrous epidemic which attacked the British expeditionary force to Walcheren in 1809 (Melville, see Ross, 1910) was due chiefly to malaria, but there can be no doubt of its importance on the Balkan front during the first World War. In 1916 about 60,000 cases of malaria occurred in the French army which at no time numbered more than 115,000 men (Ed. & Et. Sergent, 1932), and General Sarrail had to report to his government that his army was immobilized. The British (Wenyon, 1921) also were heavily infected, and it has since been revealed (Martini, 1920) that the armies of the Central Powers suffered to as great an extent.

The physical and mental development of children is affected by chronic malarial infection so that they are unable to profit to the full from the educational facilities open to them. The number of deaths due both to endemic and epidemic malaria is greatest amongst young children. According to Wilson (1936), in Tanganyika Territory babies become infected with malaria within 1–2 weeks up to a few months after birth. In areas in which, though malaria is hyperendemic, the population shows resistance to reinfection, parasites are present almost constantly in young children up to the age of 5 years, after which the infection gradually decreases in intensity. In such areas the maximum clinical effect is produced during the first 18 months of life.

Great economic loss is associated with malaria. Loss in hours of labour due to illness occurs, and in working efficiency as a result of debility and lack of stamina. The disease has proved a serious obstacle in commercial undertakings, agricultural development, the building of ports and harbours, and the development of communications.

B. *The importance of drugs in the fight against malaria*

As the malarial parasite passes part of its life in man and part in the mosquito it can be attacked either in its human host by the action of drugs, or by the destruction of its insect vector. In antimosquito campaigns the attack is usually made upon the aquatic phase of the life cycle of the insect, either by the destruction of the larvae by methods such as oiling the surface of the water or dusting with Paris green, or by the drainage of breeding places

and the hastening of the flow of sluggish streams by canalization. Though these methods give excellent results they require constant supervision. They are expensive and only possible in towns and other areas where the benefits accrued are commensurate with the cost. In some more enlightened rural districts antimosquito measures can be carried out with the active co-operation of the inhabitants, but in other parts the people are too backward, and too primitive in their ways of life, to take part in or appreciate such work. In many rural areas and in tracts of undeveloped or partially developed country, therefore, antimosquito measures are impracticable. Even in areas where the most rigid mosquito control is exercised, malaria, though greatly reduced in frequency, is not completely eradicated. Efficient drug treatment is therefore required not only to cure clinical symptoms and save life, but also as a means of combating the disease as a whole.

Quinine has been used for the treatment of malaria since early in the seventeenth century, but although the debt which mankind owes to it cannot be overestimated it has certain drawbacks. Though in most cases it has a rapid effect upon the clinical symptoms and causes the disappearance of parasites from the peripheral blood it fails, in a large proportion of cases, to sterilize the host of his infection. Thus treatment with quinine of infections of *Plasmodium vivax* and *P. malariae* is followed by relapse in up to 50 % of the cases treated (League of Nations, 1937). Though it has a parasiticidal action upon the gametocytes of *P. vivax* and *P. malariae*, its action upon the gametocytes of *P. falciparum* is only slight. It has no true causal prophylactic action against malarial infection, for it has been proved to be without action upon sporozoites introduced into the human body.

According to Cowan (1929) only a very small proportion of the world's supply of cinchona bark, from which quinine is extracted, comes from its original source, the forests of South America, whereas over 90 % comes from the plantations of Java. India produces only 4 % of the world's supply of the alkaloid, and this represents about one-third of the amount consumed annually under present conditions by that country herself. Only a small proportion of the malaria cases in India receive treatment, and if that country were to provide quinine treatment on a scale calculated to have an effect upon her malaria problem she would require to increase her production 18 times in order to be independent of imported quinine.

Howard (1933) is of the opinion that the amount of quinine calculated by the League of Nations (1932) as necessary to meet the annual requirements of the governments of malarial countries could be supplied by the existing quinine industry. Hehir (1927), however, has pointed out that it is the price of quinine, not shortage of supply, which places it beyond the reach of millions of sufferers; and the Public Health Commissioner with the Government of India (1935) stresses that the greatest difficulty, apart from distributing the drug, is to place it at the disposal of the villager at a price he can afford to pay.

Past experience has shown that the success of military campaigns in malarial country depends upon adequate prophylactic treatment with quinine of the troops taking part. In this respect, therefore, quinine can be regarded as an important munition of war.

2. CHEMOTHERAPY AND MALARIA

Efforts to synthesize antimalarial compounds more efficient than quinine and less expensive have been made for many years. Quinine shortage during the last war led to a great interest in this field of research in Germany, and their efforts have been rewarded by the synthesis of two useful antimalarial compounds, plasmoquine[1] and atebrin.

Plasmoquine has practically no effect upon the trophozoites of *P. falciparum*, but acts to some extent on the trophozoites of *P. vivax* and *P. malariae*. It acts upon the gametocytes of all three species, but its chief value is its gametocidal action upon *P. falciparum*. In association with quinine or atebrin, or given after a course of treatment with either of these drugs, it reduces the relapse rate in benign tertian and quartan malaria, and apparently in malignant tertian malaria (League of Nations, 1937).

Atebrin has an action similar to quinine, but appears to be slightly more effective in preventing relapses (League of Nations, 1937).

Plasmoquine requires to be given under medical supervision, as toxic symptoms, sometimes severe, may occur. Atebrin, though much less toxic, may give rise to toxic symptoms in individuals, and frequently causes a yellow coloration of the skin of the patient.

Though these compounds are useful, neither constitutes the ideal antimalarial drug.

The attributes necessary to the ideal antimalarial drug are many. It should be cheap, for malaria is frequently associated with great poverty. An expensive drug, however efficient, would be beyond the means of most malarial sufferers unless heavily subsidized by the governments concerned. It should be non-toxic, without cumulative toxic effect, so that it can be safely taken without medical supervision. This is necessary if the drug is to reach the masses of malarial sufferers, who may live far from doctors and clinics, and the drug must be distributed through post offices, police posts, schools and local headmen. Treatment should preferably be by mouth, and of short duration. The drug should be palatable and produce an immediate improvement in the clinical condition, since it is difficult to persuade people of a low standard of education to take an unpleasant drug or one from which no immediate benefit is derived. It should give rise to no secondary symptoms, such as discoloration of the skin. It should be stable under widely varying climatic conditions, and non-

[1] This compound is called Plasmochin in German, but it is generally referred to as Plasmoquine in English scientific literature, under which name it has been marketed in England. I have therefore used the English name throughout this paper.

hygroscopic. It should act upon all species and all races of malaria, and upon all stages of the life cycle.

3. Requirements for research upon the chemotherapy of malaria

One of the difficulties at first experienced in research upon the chemotherapy of malaria was the need for some suitable means of testing the antimalarial action of the compounds synthesized, for it is not possible to transmit malaria of man to animals. It is therefore necessary to use some other species of *Plasmodium* upon which drugs will have a similar action to that upon human malaria. Since the search for antimalarial drugs may involve the testing of hundreds, or even thousands of compounds, the animal to be infected with *Plasmodium*, and upon which the drugs are to be tested, should be inexpensive, easy to house and to handle, cheap to feed, healthy under laboratory conditions, and free from infection with *Plasmodium* when bought from the dealer. An important consideration in the choice of an animal is that it should be small, for the amount of drug is given in proportion to the body weight, and the yield of new synthetic compounds is often small. Samples of 100–200 mg. are frequently submitted for test by chemists.

Canary hens (*Sirenus canarius*) fulfil these conditions. Though the cock birds are expensive, being valued for their song, hens can be bought cheaply. Species of avian malaria can be transmitted to the canary; and these birds, experimentally infected with *Plasmodium relictum*,[1] have been used most frequently to test the antimalarial activity of new compounds. The Roller variety of canary is particularly suited to this work, as it is smaller than most other varieties, and it is always used in this Institute.

4. The development of the use of avian malaria in chemotherapy

The discovery by Koch (1899), in collaboration with R. Peiffer, that canaries were susceptible to infection with *Proteosoma* (=*Plasmodium*) of sparrows, formed the foundation of experimental research upon avian malaria by the introduction of a host well suited to laboratory conditions. The infection was transmitted by the injection of diluted, infected blood into the breast muscle of the bird, and in over 100 canaries Koch did not find one which was insusceptible to infection. The incubation period was about 4 days. The

[1] The correct specific name of the first species of avian malaria to be described has been the subject of some controversy. Sergent, Ed., Sergent, Et. & Catanei (1929*b*) are of the opinion that *relictum* is the correct name, whereas Manwell (1935), on the grounds that *praecox* has been used more frequently, advocates the acceptance of this specific name. But the use of *praecox* for the parasite of avian malaria is not free from ambiguity, as this name has been applied by some workers to the parasite of malignant tertian malaria of man. On this account I have used *relictum* throughout this paper, but a perusal of the original papers to which I refer will show that some authors have used *praecox* and others *relictum*.

height of the infection was reached between the 8th and the 10th day, and by the 14th day parasites could no longer be found in the peripheral blood. In order to discover whether birds which had recovered from the infection were immune to reinfection Koch inoculated twelve birds with heavily infected blood 4 weeks after the previous infection. Ten of these birds remained free from infection, whilst the remaining two developed slight infections. From this he deduced that recovery from infection was complete and accompanied by immunity to reinfection. That his conception was not correct was proved by Wasielewski (1902) who was able to find parasites, after prolonged search, in the blood of canaries which had recovered from the acute infection. He found that the blood of a bird in which no parasites had been seen for 11 days previously produced an infection when inoculated into a clean bird, and that therefore the apparent disappearance of parasites from the peripheral blood was not a proof of complete recovery.

Further experiments led Wasielewski to conclude that in chronic infections the number of parasites present might be too few to produce an infection in every bird inoculated. The results of other workers have shown that the amount of blood used in the inoculum, and therefore the number of parasites introduced, is important. Boyd (1925) was unable to obtain infections when 100 parasites, calculated by dilution methods, were introduced, and of birds inoculated with 1000 parasites 71·4 % failed to become infected. Manwell (1930) advocated the injection of at least 250 mg. of blood, as smaller amounts did not always produce an infection. Alternatively, two or three birds should be inoculated from a chronic bird, which is the practice in this laboratory. Coulston & Manwell (1940) have, however, obtained infections of *Plasmodium circumflexum* by the inoculation of a single red blood corpuscle containing a single parasite.

The reinoculation with heavily infected blood of chronic birds in which no parasites had been found for varying periods, was found by Wasielewski (1902) to produce very mild infections. Moldovan (1912) found that so long as the infection persisted no acute attack resulted from reinoculation, but on complete recovery, as demonstrated by the absence of parasites on repeated search, the susceptibility to reinfection was restored and an acute attack developed. Descriptions of complete recovery are very rare. Mazza (1924) found that two canaries which had been infected with *P. relictum* 3 years and 3 months previously and which had been proved free from infection by the failure of their blood to infect clean birds, developed normal, acute attacks on reinoculation. Gingrich (1932) described one canary, previously infected with *P. elongatum*, whose blood proved non-infective to a normal bird when 0·25 c.c. was injected intravenously, and which on reinoculation suffered from a second acute infection. A chronic infection succeeded this second attack, and no further attack developed on reinoculation 4 months later.

With rare exceptions, once infection has been established in birds, parasites

persist throughout life, for they have been found up to 8 years and 3 months after infection (Bishop, Tate & Thorpe, 1938).

The fact that parasitized blood fails to produce a normal acute attack when it is injected into a bird carrying a latent infection of the same strain of *Plasmodium* is used to determine whether an infection has been eradicated or merely suppressed by the action of a drug. This is called the *Immunity Reaction* by the Sergents (1921*a*). An alternative method of proof is by the injection of blood from the bird believed to be cured of its infection into clean birds. This is called *Isodiagnosis* by the Sergents.

5. The cinchona alkaloids and avian malaria

Wasielewski (1908) first suggested that *Plasmodium relictum* might provide a means of studying the therapeutic action of drugs upon malaria, but his own experiments dealt only with the action of quinine hydrochloride upon parasites of *P. relictum* in vitro. The resistance of the parasites outside the body of the host suggested the possibility of such experiments to him. At room temperature most of the parasites were killed by quinine 1/2000 in 30 min., and all were killed by 1/1200 in 5 min. Anschütz (1910) found that quinine had only a slight action, in vitro or in vivo, on the parasites, but the results of well-planned experiments by Kopanaris (1911) did not confirm this. He found that parasites were no longer infective after 15 min. treatment with quinine hydrochloride 1/1200 in vitro, though quinine 1/2000 acting for 30 min. was without effect. As canaries tolerated an intramuscular injection of 0·5 c.c. of 0·5 % solution of quinine hydrochloride he studied its effect upon *P. relictum* experimentally inoculated into canaries. The drug was injected on the 6th day following the inoculation of the bird with parasitized blood, when parasites were first numerous in the peripheral blood. A diminution in the number of parasites as compared with the untreated control birds was observed. The injection of 0·5 c.c. of 0·25 % quinine on 3 consecutive days gave almost similar results. The gametocytes, particularly the macrogametocytes, were more resistant than the asexual forms. Kopanaris thus showed that quinine given curatively (i.e. after the appearance of the parasites in the peripheral blood) had a definite action upon *P. relictum* in canaries. He was unable to discover any prophylactic action of the drug.

In experiments similar to those with quinine Kopanaris then studied the effect upon avian malaria of salvarsan, which had been found by Werner (1911) to have some effect upon benign tertian malaria of man. Although the birds appeared to tolerate the drug fairly well it was not only without antimalarial action, but the infections were heavier and lasted longer than in the untreated controls. He did not believe that this was due to chance, but suggested that the toxicity of the drug prevented the development of the normal defence mechanism of the bird. He found that atoxyl had no effect upon *P. relictum* in canaries.

The Sergents (1921, 1921*a*, *b*) studied the effect of quinine injected subcutaneously into canaries infected with *P. relictum*. Daily doses of 0·7 mg., given from the day on which the bird was inoculated with citrated, infected blood for a period corresponding to that of the attack in the controls, suppressed the infection but did not eradicate it, for in some birds a few parasites were found and others, apparently negative, were proved to be resistant to reinoculation. In some birds the infection which had been latent during quininization became acute when treatment ceased. Quinine was ineffective if given before inoculation with parasites. A large dose (2·8–3·5 mg.) of quinine given within 24 hr. following inoculation caused a delay of 2–7 days in the development of the attack. Small doses of quinine (0·7 mg.) administered daily for 6–7 days (i.e. during the normal period of incubation) delayed the appearance of parasites for 8–9 days, but were without effect upon the virulence or duration of the subsequent infection. When a large dose (2·5–4 mg. given in two or three fractions) was given at the acute stage of the infection parasites disappeared from the peripheral blood in eleven out of twelve birds in less than 24 hr.

The Sergents concluded that prophylactic treatment with quinine before inoculation with parasites had no effect upon the infection—it should be begun on the day of inoculation.

It was thus established, principally by the work of Kopanaris and the Sergents, that the well-known therapeutic action of quinine upon malaria of man extended also to avian malaria, but although quinine was able to delay the appearance of parasites in the peripheral blood, or to cause a temporary disappearance of parasites in infections already established there, it was unable to eradicate the infection.

That quinine did not prevent the development of immunity to superinfection was shown by Lourie (1934*a*), who found that, in birds in which an attack of *P. cathemerium* had been suppressed but not sterilized by quinine, immunity to superinfection was as strong as in those in which the primary attack had taken a normal course.

In collaboration with Catanei the Sergents (Sergent *et al.* 1923) found that cinchonidine hydrochloride was a little more toxic to canaries than quinine, and in non-toxic doses given at the acute stage of the infection was without effect upon *P. relictum*, though small daily doses given over a long period, beginning with the day of inoculation, delayed the appearance of parasites; but its action was less marked than that of quinine. The authors (Sergent *et al.* 1924) later found that while cinchonine was a little more toxic to canaries than quinine its action upon the parasites was inferior; but nevertheless it had a definite antimalarial action.

In addition to quinine Boyd (1926) studied the action of new, synthetic derivatives of quinine upon infections of *P. relictum* in canaries, with the aim of finding a more effective antimalarial drug. In some of his experiments he used oral injection of the drug, by means of a fine catheter tube attached to

a syringe, in place of the intramuscular or subcutaneous mode of injection used by previous workers. Brünn (1926) also used the peroral method of administering quinine to canaries. The quantity of solution given by Boyd was 0·25 c.c., and variations in the dose were made by varying the concentration of the drug, not the volume of the fluid. The results of his experiments with quinine confirm those of the Sergents. None of the synthetic compounds showed any definite antimalarial action.

Morgenroth, Abrahams & Schnitzer (1926) also confirmed the Sergents' observations, and they discovered that hydroquinine hydrochloride was even more effective than quinine in delaying the appearance of parasites. Thus they found that quinine and hydroquinine bore the same relationship to each other in their action upon avian malaria as Giemsa & Werner (1912) and MacGilchrist (1915) had found in malaria of man.

Morgenroth and his co-workers also found that optochin (ethyldihydrocupreine) had a definite action against *P. relictum*.

6. The development of large-scale research upon the antimalarial activity of new, synthetic compounds

Whereas until 1926 substances known to be active upon malaria of man were proved to be active also upon malaria of birds, the antimalarial properties of plasmoquine, a new, synthetic compound, were discovered by tests upon avian malaria, and the activity thus evinced was proved later to extend to malaria of man. This compound was the result of many years of close collaboration between chemists and biologists at the Bayer laboratories, Elberfeld, Germany, and of a precise method devised by Roehl (1926) for testing the antimalarial activity of compounds.

The discovery of this synthetic, antimalarial drug aroused wide interest in research of this kind, and the search for other synthetic compounds with antimalarial properties was pursued on a large scale, principally in England, France, Germany and Russia.

A. *The development of research upon the chemotherapy of malaria at the Molteno Institute, University of Cambridge*

In 1927 the Medical Research Council of Great Britain established a biological unit for testing the antimalarial action of new synthetic compounds, the tests being made by Dr P. Tate and Miss Vincent at the Molteno Institute under the direction of Prof. D. Keilin, F.R.S., and, in the early days of the unit's history, by Dr J. W. S. Macfie at the London School of Hygiene and Tropical Medicine.

In work of this kind, where, as in the case of plasmoquine, the production of an effective compound may be the result of many years of work and may involve the testing of large numbers of compounds, continuity in the biological work is essential, since the tests, to be reliable, must be made under precisely similar conditions and upon the same strain of parasite. The biological unit at

the Molteno Institute has continued its work without interruption from December 1927 until the present day.

Several laboratories have experienced difficulty in keeping canaries healthy when large numbers are involved, and their work has been interrupted by the outbreak of epidemics. In the Molteno Institute the birds are kept in a separate room, and the windows and doors are screened to prevent the entrance of mosquitoes. Unpainted metal cages of a simple design, which are easy to clean, are used. The cages are arranged on metal tubular shelving which avoids the accumulation of dust, and of faeces and seeds cast out of the cages. By this method large numbers of cages can be stocked in a moderate-sized room. The birds are kept in individual cages which are cleaned frequently, and always sterilized before re-use. The cages are kerosened along all cracks at intervals of a few weeks, in order to control red mite which attacks cage birds. A balanced diet is given, with green food twice a week. The bird room is kept scrupulously clean, all dust, waste seed, etc., being removed from the floor daily by a vacuum cleaner. To prevent the introduction of disease into the stock new birds are isolated in a separate room, in separate cages, for at least a fortnight, after which only those which appear healthy are admitted to the stock. Canaries have been kept in this Institute in large numbers since 1928, and no outbreak of disease has occurred amongst the stock, though disease has broken out amongst new arrivals during their period of isolation.

No mosquito work is done in the bird room, birds required for experiments with mosquitoes being removed to another room.

Although the routine tests for antimalarial activity are made upon infections transmitted to the birds by the inoculation of infected blood, mosquito-borne infections are also used (p. 13). Tate & Vincent (1932*b*) were the first workers in England to infect *Culex pipiens* with *Plasmodium relictum*. The difficulty experienced by MacGregor & Lee (1929) in getting this mosquito to gorge on birds under experimental conditions was overcome by Tate & Vincent (1932*a*, 1936) by exposing the females to light for prolonged periods before putting them into the dark to bite. They found that of females of *Culex pipiens* kept continuously in the dark only about 3 % gorged, whereas after exposure to light 66–90 % gorged.

An autogenous race of *C. pipiens* was used by Tate & Vincent in place of the normal, anautogenous strain in experiments upon insect-borne infections of *Plasmodium relictum*. The autogenous race is easier to cultivate in the laboratory than the anautogenous race, for it will oviposit without a blood meal, does not hibernate, and can be fertilized in a confined space. Although the strain can be kept indefinitely without blood meals the females will gorge when required. Large breeding cages are not necessary, and no illumination is needed to induce the females to gorge. Such an autogenous race is part of our equipment for insect transmission experiments.

Tate & Vincent (1934*a*) found no difference in susceptibility in the autogenous and anautogenous strains of *Culex pipiens* to the same strain of

Plasmodium relictum, nor in their ability to transmit the infection. But two strains of *P. relictum*, a German strain, the gift of the late Dr Roehl, and an Algerian strain, the gift of Prof. Ed. Sergent, differed in the infection rate produced in the mosquitoes. Thus 89 % of the mosquitoes gorged upon birds infected with the Algerian strain of *P. relictum* became infected, whereas only 43 % became infected with the German strain. The two strains were found to differ markedly in their tendency to form gametocytes, but differences in the infection rate of the two strains could not be explained merely by the number of gametocytes present in the bird on which the mosquitoes had gorged.

In the German strain of *P. relictum* the appearance of gametocytes is irregular, and at some periods of the year they are rare, whereas in the Algerian strain large numbers of gametocytes are produced regularly. The routine tests for antimalarial activity upon infections induced by blood inoculation are made upon the German strain, but the Algerian strain is used for tests upon mosquito-induced infections at periods of the year when gametocytes are rare in the German strain. No difference in their reaction to drugs has been observed in the two strains.

B. *The characteristics of the German strain of* Plasmodium relictum *in canaries*

In the German strain of *P. relictum*, which is used in the Molteno Institute for the routine tests for antimalarial activity of new drugs, the incubation period, i.e. the period between the inoculation of infected blood into the breast muscle of the bird and the appearance of parasites in the peripheral blood, is usually 5–6 days. When the infection is repeatedly passaged from heavily infected birds the incubation period is remarkably constant, but if birds with chronic infections are used as the source of the inoculum the incubation period is irregular, and the parasites may not appear before the 10th to 20th day. After the appearance of the parasites in the peripheral blood on the 5th or 6th day their number increases rapidly, and the infection normally reaches its peak between the 8th and 12th days. The number of parasites then decreases rapidly, and by the 14th to 21st day few or no parasites are to be found in a 3 min. examination. That such birds, though apparently negative, still harbour parasites in their blood can be demonstrated by inoculating clean birds with their blood, when infections will be produced (p. 7), but after an incubation period longer than normal. The average infection rate appears to lie between 60 and 175 parasites per 1000 corpuscles, but birds with heavier or lighter infections are not rare. It is seldom that a bird dies as the result of the infection.

The incubation period in infections induced by the bite of infected mosquitoes, or by the inoculation of ripe sporozoites from teased-up salivary glands, is less regular, both in the German and the Algerian strain, than in infections induced by the inoculation of blood from heavily infected birds.

C. *The technique used in testing the antimalarial activity of compounds*

A general survey of the literature of the subject emphasizes that if tests upon avian malaria are to be reliable and comparable one with another it is necessary that the same species and strain of *Plasmodium* should be used throughout, and the same technique of infection and of administration of the drugs should be adhered to.

It is our practice to test all compounds first upon infections induced by the inoculation of infected blood, but those which show definite antimalarial activity are tested further upon sporozoite-induced infections. The inoculation is made into the breast muscle, the inoculum consisting of 0·3 c.c. of a mixture of heavily infected blood and citrate-saline solution. When blood is required in quantity, as is the case when a series of birds is to be inoculated, the blood is taken from the leg vein, but when small quantities are required, as for a blood film, it is taken from the toe. Many workers take blood from the wing vein, but Tate & Vincent found that severe bleeding, with the formation of haematoma under the skin of the wing, frequently occurred when the wing vein was pricked, whereas bleeding from the toe was slight and easily controlled.

The drug is given according to Roehl's method (p. 15), by injection through a fine catheter tube attached to the end of a graduated syringe. Vincent found that if the catheter tube was warmed in water it would pass more easily into the stomach of the bird. The beak of the bird is held open by forceps and the catheter tube passed down the oesophagus into the stomach. Tate & Vincent found that if 0·5 c.c. of solution of drug per 20 g. of body weight of bird was given at each dose, in place of the 1 c.c. given by Roehl, regurgitation of the drug was much less frequent.

When the *maximum tolerated dose* has been found it is given in six successive daily doses, the first dose being given 4 hr. after the inoculation of the bird with parasites. Birds are examined for parasites beginning on the 5th day, and the course of the infection in the treated birds compared with that in untreated birds. Each slide is examined for 3 min., and daily examinations are made until the 10th day, after which examinations are made on alternate days until the 30th day. If no parasites are found until the 30th day the birds are reinoculated with heavily infected blood to test whether the drug has sterilized them of their infection. A delay in the appearance of parasites beyond the 10th day is regarded as an indication of antimalarial activity, in accordance with Roehl's method. We have found that the delay in appearance of parasites beyond the 10th day may be variable though the dose be constant, for doses bordering upon the minimum effective dose may delay the appearance of parasites for many days in one bird but only until the 10th day in another. If the maximum tolerated dose (i.e. on six successive days) delays the appearance of parasites beyond the 10th day dilutions are tested by the same method until the *minimum effective dose* is found. The ratio of these two doses constitutes the chemotherapeutic index. This index is not strictly com-

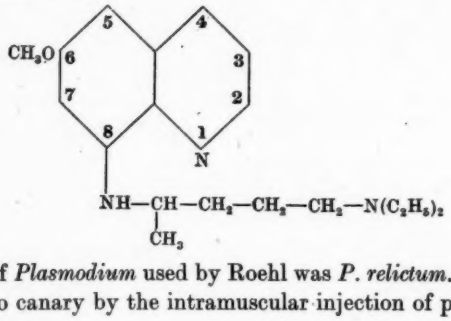
parable to that of Ehrlich. According to Ehrlich the chemotherapeutic index is the $\frac{\text{Maximum tolerated dose}}{\text{Minimum curative dose}}$. In the treatment of malaria a 'cure', i.e. sterilization of infection, may not be obtained even with the maximum tolerated dose, and the minimum dose which is considered to be significant is that which on 6 successive days will produce a delay until the 10th day in the appearance of parasites in the peripheral blood.

Although water-soluble compounds are most suitable for peroral injection by Roehl's method, with a little care insoluble compounds can also be administered, though not with the same exactitude, if they make a fine suspension in water. We have also found it possible to suspend impure compounds, which otherwise formed a tarry deposit when added to water, in solutions of gelatine and to administer them thus in suspension.

7. Plasmoquine

Previous to the discovery of plasmoquine attempts had been made to synthesize compounds which would be effective against malaria, but these syntheses consisted in variations in the constitution of quinine and no progress in malaria therapy had resulted from them. Schulemann, Schönhofer and Wingler took methylene blue as their starting point (Schulemann *et al.* 1932; Schulemann, 1932), as this compound had been shown by Guttmann & Ehrlich (1891) to have some effect upon malaria of man. When an aliphatic basic group was substituted in place of a methyl group in one of the amino groups in methylene blue, a dyestuff was produced which was proved by Roehl to be effective against avian malaria. When this basic aliphatic group was united to the quinoline nucleus by a nitrogen bond in position 8 a compound resulted which was not a dyestuff, but which had a marked effect upon avian malaria. Many different substitutions were made in the quinoline nucleus and variations in the side chain, and the compounds so formed were tested by Roehl for antimalarial action. Finally, plasmoquine was selected for further tests.

The formula of plasmoquine, N-diethylamino-isopentyl-8-amino-6-methoxyquinoline, was first published in an editorial note in the *Arch. Schiffs- u. Tropenhyg.* **32**, 382, in 1928.



The species of *Plasmodium* used by Roehl was *P. relictum*. He transmitted it from canary to canary by the intramuscular injection of parasitized blood.

He used the peroral method of injection of the drug into the stomach of the bird, for he realized that repeated injection of drugs into the muscle would not be possible if the alkaloid produced strong local reactions. A dose of 1 c.c. of drug per 20 g. of body weight of bird was given on each of 6 successive days beginning with the day of inoculation. In untreated birds parasites first appeared in the peripheral blood on the 4th–5th day. When quinine 1/200, 1/400 and 1/800 was given on each of 6 successive days the appearance of parasites was delayed until at least the 10th day, but when 1/1600 was given parasites appeared at the same time as in the untreated controls.

Roehl attached no importance to the number of days delay in the appearance of parasites beyond the 10th day though he always determined this; he considered the delay until the 10th day to be the criterion in determining whether a compound possessed antimalarial activity or not. By determining, by the oral method, the maximum dose of drug which the birds would tolerate (referred to hereafter as the M.T.D. or maximum tolerated dose), and the lowest dose which would produce the significant delay in the appearance of parasites (referred to hereafter as the M.E.D. or minimum effective dose) the range of activity of a compound, or chemotherapeutic index, was determined. According to Roehl the M.T.D. of quinine was 1/200 and the M.E.D. 1/800, giving an index of 1/4. With plasmoquine the M.T.D. was 1/1500 (0·66 mg. per 20 g. body weight of bird) and the M.E.D. 1/50,000 (0·02 mg. per 20 g. body weight of bird), giving an index of approximately 1/30 as compared with 1/4 for quinine. Whereas plasmoquine 1/50,000 delayed the appearance of parasites until at least the 10th day, some of the stronger solutions caused a delay of up to 6 weeks, and sometimes no parasites were found after even longer periods. Roehl determined whether these birds were sterilized of their infections by the immunity reaction and by isodiagnosis. Though some birds were sterilized of their infections this was not assured even with the biggest doses.

If a single large dose of plasmoquine was given when the parasites had already appeared in the peripheral blood Roehl noticed that within 48 hr. all the large parasites and gametocytes vanished and only small trophozoites remained. These were abnormal in form, and later they too disappeared. But parasites reappeared ultimately, for sterilization was never obtained by curative treatment.

Roehl discussed the question whether plasmoquine had a direct lethal action upon the parasites or whether it acted indirectly by the formation of a protective substance in the host. When canaries were treated with plasmoquine (1 c.c. of 1/1500 per 20 g. of bird) and inoculated with *P. relictum* 2 hr. later a normal infection resulted, but if the birds were treated with the same dose of plasmoquine 6 hr. after inoculation the drug had a definite action upon the course of the infection. If the action of plasmoquine were indirect it is hardly probable that any protective substance formed would have disappeared or lost its antiplasmodial properties within 2 hr. Roehl therefore believed its action to be direct.

By Roehl's method therefore the prophylactic, not the curative, action of a drug is assessed, his criterion of activity being the power to delay the appearance of parasites until the 10th day after inoculation and the beginning of treatment. The further basis of comparison necessary to compare compounds with marked antimalarial activity is provided by their chemotherapeutic indices.

Reports that plasmoquine, so effective against avian malaria, was also effective against malaria of man were soon forthcoming (Sioli, 1926; Mühlens, 1926; Roehl, 1927; Schulemann & Memmi, 1927). Schizonts of quartan malaria proved more resistant to plasmoquine than those of benign tertian malaria. Upon schizonts of malignant tertian malaria plasmoquine had little effect, but upon the crescents its effect was very marked. The importance of plasmoquine is not that it replaces quinine, but that by its action upon gametocytes, particularly those of *P. falciparum* upon which quinine has little effect, it offers an additional method of attack upon malaria.

Many descriptions of the action of plasmoquine upon avian malaria followed Roehl's publication, but the methods and species of parasite used have differed, so that it is difficult to compare the results.

The doses given by Hegner & Manwell (1927) were irregular and not adjusted to the body weight of the bird. The species of parasite was not stated, but according to Manwell (1930) it was *P. cathemerium*. Infection was not prevented either by large doses of plasmoquine given for 5 or 6 days following inoculation, or by smaller doses given from the 4th to the 11th day, though the drug delayed the appearance of parasites in the former case and prevented acute infection in the latter.

Manwell (1930) found that plasmoquine reduced the number of parasites of *P. relictum* in the peripheral blood during treatment to a lower level than quinine.

Russell (1931*a*, *b*) studied the effect of plasmoquine upon *P. cathemerium* in canaries but did not vary the size of the dose according to the body weight of the bird. Birds were given a 7-day course of treatment with plasmoquine, and inoculated with parasitized blood on the 3rd, 4th, 5th, 6th or 7th day from the beginning of treatment. None of the birds became infected, with the exception of those which were inoculated 5 hr. after the final treatment. All birds treated in a similar way with quinine hydrochloride became infected. This 100% sterilization of birds with plasmoquine, whether with seven daily doses or one single dose, is not in agreement with the result of Hegner & Manwell (1927) who were unable to sterilize infections of *P. cathemerium* with large doses of the drug, or of Manwell (1930) who found this species the most resistant to plasmoquine of the species of avian malaria which he treated.

Tate & Vincent (1933*b*) studied the effect of plasmoquine simplex upon the German strain of *P. relictum* (pp. 12–13). Their results were strictly comparable with those of Roehl, if allowance is made for the different volume of solution of drug administered. Whereas Roehl found the M.T.D. to be 1/1500 (i.e. approx.

0·66 mg. per 20 g. of body weight), Tate & Vincent found that few birds would tolerate this dose on 6 consecutive days, and they considered the highest *safe* dose to be 0·32 mg. A dose of 0·02 mg. was still markedly effective as Roehl had found. In recent experiments I have confirmed that 0·32 mg. is the highest safe dose when given on 6 consecutive days, and have also found that $6 \times 0{\cdot}01$ mg. had still a significant effect in three out of four birds. The criticism which might be levelled at Roehl's otherwise excellent paper is that he does not state the number of birds treated nor the proportion sterilized. This does not apply to the work of Tate & Vincent. Their results indicate that plasmoquine was almost equally effective throughout the range of doses tested, i.e. 0·64–0·02 mg., and that in birds not sterilized of their infection the appearance of parasites was delayed until at least the 20th day. The number of birds sterilized was sixteen out of thirty-three. In one bird a single dose of 0·64 mg. given 4 hr. after inoculation was sufficient to sterilize it of its infection, and the authors considered that the time at which the drug was given in relation to the time of inoculation was important in the efficacy of the treatment. Plasmoquine given prior to inoculation was found to have no prophylactic action, as Roehl and Russell had discovered.

8. THE USE OF *HAEMOPROTEUS* IN THE STUDY OF ANTIMALARIAL COMPOUNDS

Gody & Lacorte (1928), impressed by the selective action of plasmoquine upon the gametocytes of *Plasmodium falciparum*, studied its action upon *Halteridium* (=*Haemoproteus*) in the pigeon. In this genus only the gametocytes are found in the peripheral blood, for schizogony takes place in the endothelial cells, particularly of the lung. The authors found that in birds treated with plasmoquine the gametocytes disappeared rapidly from the peripheral blood but reappeared after 4 or 5 days. From this they inferred that the action of plasmoquine was gametocidal, not schizontocidal. When pigeons free from *Halteridium* were injected with an emulsion of *Lynchia lividicolor* rich in sporozoites of *Halteridium*, and an hour later received 0·01 g. of plasmoquine, they did not become infected, but if the drug was withheld for 24 hr. parasites appeared as in the controls. The authors concluded that sporozoites, like gametocytes, were destroyed by plasmoquine, but that they underwent a change within 24 hr. from their entry into the vertebrate host which rendered them resistant to the drug. The evidence upon which these pigeons were considered to be free from natural infection was not given.

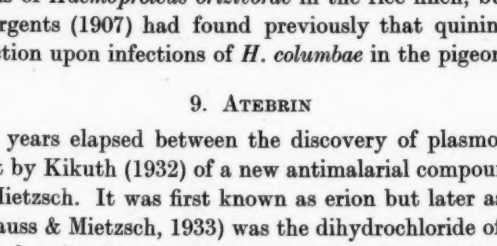
Collier & Krause (1929) pointed out that whereas it is possible, by Roehl's method, to test the preventive action of a drug upon infections of *Plasmodium relictum* in canaries, it is impossible, owing to the briefness of the acute infection, to test its curative action satisfactorily. If a drug is given at the height of the infection and parasites disappear shortly afterwards it is impossible to determine whether this is due to the action of the drug or to natural recovery. The presence of parasites in the peripheral blood during the sub-

sequent chronic infection is too sporadic to form a basis for assessing the chemotherapeutic action of drugs. Though there may be differences in intensity in infections of *Halteridium* (=*Haemoproteus*) in the rice finch (*Padda orizivorae*), the infection runs a fairly even course over a long period. The authors therefore advocated the use of this parasite for tests upon the curative action of drugs. They failed, however, to point out that whereas in infections of *Plasmodium* the action of a drug can be observed on the gametocytes and the schizonts, as both are present in the peripheral blood, in infections of *Haemoproteus* its action can be observed upon the gametocytes alone, and any action upon the schizonts can be inferred only from indirect evidence. Plasmoquine caused the disappearance of *Haemoproteus* from the blood of rice finches for at least 14 days.

According to Fourneau, Tréfouel, J. M. & Mme. Bovet & Benoit (1931), plasmoquine, methylene blue and quino-stovarsol all had a therapeutic action upon infections of *Haemoproteus orizivorae* in the rice finch, but quinine had none. The Sergents (1907) had found previously that quinine was without therapeutic action upon infections of *H. columbae* in the pigeon.

9. Atebrin

Not many years elapsed between the discovery of plasmoquine and the announcement by Kikuth (1932) of a new antimalarial compound synthesized by Mauss & Mietzsch. It was first known as erion but later as atebrin. This compound (Mauss & Mietzsch, 1933) was the dihydrochloride of 2-methoxy-6-chlor-9-α-diethyl-amino-δ-isopentylaminoacridine. In atebrin, as in quinine



and plasmoquine, the methoxy group is in the *para* position to the heterocyclic nitrogen. The basic side chain is similar to that of plasmoquine and is united to the nucleus by an amino group, but the position of its side chain in relation to the heterocyclic nitrogen resembles quinine, not plasmoquine. Mauss & Mietzsch pointed out that atebrin was only one compound of a series possessing antimalarial properties, and many changes could be made in its structure, such as substitutions in the methoxy group, the replacement of the Cl atom by other halogen atoms, or substitutions in, or shortenings of, the basic side chain as in the plasmoquine series, without the total disappearance of antimalarial activity in the resulting compounds.

Kikuth (1932, 1933) found that the chemotherapeutic index of atebrin, when tested upon *Plasmodium relictum* in canaries by Roehl's method, was the same as that of plasmoquine (1/30). Atebrin was, however, less toxic than plasmoquine; and as the M.E.D. of atebrin was only 1/3000, as compared with 1/50,000 for plasmoquine, it was, in absolute terms, about 15 times less active than plasmoquine, but it was 4 times more active than quinine. In the strongest doses Kikuth found that it did not produce such a long delay in the appearance of parasites as plasmoquine, and in this respect its action resembled more closely that of quinine than plasmoquine. When given curatively it caused a temporary disappearance of parasites from the peripheral blood and failed to sterilize the infection. The response of *P. elongatum* and *P. circumflexum* was similar to that of *P. relictum*.

Kikuth, using Roehl's method with infections of *Plasmodium* in canaries, found it possible to show only quantitative differences in the therapeutic action of antimalarial compounds; qualitative differences such as schizontocidal and gametocidal properties were not revealed. But with infections of *Haemoproteus orizivorae* he believed it possible to demonstrate such differences if they existed. Plasmoquine caused a disappearance of the gametocytes of *Haemoproteus* from the peripheral blood for a varying length of time, but since they reappeared soon after treatment had ended Kikuth concluded that the drug had little or no effect upon the schizonts in the internal organs from which, presumably, the gametocytes arose. Atebrin, like quinine and unlike plasmoquine, had no action upon the gametocytes, but when it was given in addition to plasmoquine the gametocytes either failed to reappear or their appearance was delayed for a much greater time than when plasmoquine alone was used. Kikuth therefore deduced that the action of atebrin was schizontocidal.

Coatney (1935) found that atebrin produced morphological changes in the gametocytes of *Haemoproteus columbae* of the pigeon, and removed them slowly from the peripheral blood. It also appeared to inhibit the growth of the young gametocytes. He failed to find any evidence of a schizontocidal action, there being little difference in the time of reappearance of the gametocytes after treatment with atebrin as compared with plasmoquine. He did not, like Kikuth, give the two drugs in a combined treatment.

Sioli (1932) and Peter (1932) reported upon the successful treatment of cases of induced and naturally acquired malaria in man with atebrin. In benign tertian malaria Peter found that atebrin acted first upon the rings, then upon the schizonts, and lastly upon the gametocytes. Its action upon the rings of *Plasmodium falciparum* was rapid, but the gametocytes were not affected.

Results of experiments by Tate & Vincent (1934*b*) on the action of atebrin upon *P. relictum*, transmitted to canaries by blood inoculation, were in accord with those of Kikuth. They too found that, when given prophylactically by Roehl's method, though it delayed the appearance of parasites in the peripheral blood it was unable to prevent infection. It was without action upon gametocytes.

10. The action of synthetic drugs upon gametocytes

Mühlens (1926) and Roehl (1927) had observed that plasmoquine destroyed the crescents of *P. falciparum*. Barber & Komp (1927), and Barber, Komp & Neuman (1929) found that small doses of the drug affected the viability of the crescents, rendering them non-infective to mosquitoes though not necessarily incapable of exflagellation.

That plasmoquine and plasmocide[1] had a similar action upon the gametocytes of *P. relictum* was observed by Kritschewski & Pines (1934). They found that the gametocytes ceased to be infective to *Culex pipiens* within 24 hr. of the treatment of their host (*Spinus spinus*) with plasmoquine 1/1000 or plasmocide 1/1500, whereas dilutions of 1/4000 or 1/8000 were ineffective. The authors suggest that all quinoline derivatives may possess the power to break the chain of the man-mosquito developmental cycle of the parasite. Bovet, Benoit & Altmann (1934), however (p. 39), are of the opinion that the gametocidal action of quinoline compounds depends on the length of the side chain at position 8 on the quinoline nucleus. In higher homologues of the series there is a decrease in the gametocidal properties and an increase in the schizontocidal properties. It is therefore improbable that all quinoline compounds would be able to prevent the infectivity of gametocytes to mosquitoes as Kritschewski & Pines suggest.

When blood containing ripe microgametocytes of *Plasmodium* or *Haemoproteus* is placed in a moist chamber and incubated at 37° C. for 5 min. or longer exflagellation occurs. Mochkovski (1935) studied the effect of compounds of the quinoline and acridine series upon exflagellation. Birds rich in gametocytes of *Plasmodium relictum* or *Haemoproteus* were treated with the drug and the blood tested 18–24 hr. later for exflagellation. Whereas several members of the quinoline series (e.g. plasmoquine, plasmocide, and Fourneau 736, a 6-methoxy-8-aminoquinoline compound with a side chain

$$—CH(CH_3)CH_2—CH_2—N(C_2H_5)_2$$

at 8) inhibited exflagellation, eight acridine compounds, including atebrin, did not.

Kikuth (1938) found that plasmoquine, given to a bird infected with *Plasmodium cathemerium* 1 hr. before the blood was drawn, prevented the exflagellation of ripe microgametocytes though the gametocytes had undergone no noteworthy change in appearance. By testing the inhibitory action of drugs upon exflagellation he believed that differences in antimalarial action could be detected between compounds which by Roehl's criteria of activity appeared to be almost identical. Several factors led to variations in the results of these exflagellation tests, so that he found it necessary to repeat the experiments many times and to make many controls.

Kikuth (1938) found that certuna (cilional), a compound described as a

[1] mula of plasmocide is 6-methoxy-8-diethylamino-propylaminoquinoline (p. 38).

dialkylamino-oxyquinolylaminobutane, prevented exflagellation in *P. cathemerium* in a dilution of 1/200,000 as compared with 1/12,000 for plasmoquine. The toxicity of certuna to canaries was similar to that of plasmoquine, but it was only half as active when tested by Roehl's method. Against infections of *Haemoproteus* in the rice finch its activity was double that of plasmoquine. Kikuth concluded that certuna had specific gametocidal properties.

Opinions upon the value of certuna in the treatment of malaria vary, though all agree that, in the doses prescribed, it is well tolerated. Its gametocidal action upon crescents of *Plasmodium falciparum* was considered by Mühlens (1938) to be superior to that of plasmoquine, and by Missiroli & Mosna (1938) to be equally effective. As, according to their experience, much larger doses of certuna than of plasmoquine were necessary to destroy the gametocytes of *P. falciparum*, and as it acted more slowly than plasmoquine, Chopra, Das Gupta & Sen (1938) saw no justification for the use of certuna in preference to plasmoquine. Decourt, Belfort & Schneider (1939) considered it to be less effective than plasmoquine or rhodoquine (p. 37). Certuna, even in large doses, did not, in Sioli's (1938) experience, cure infections of *P. vivax* experimentally induced in cases of general paralysis of the insane, though it caused a temporary suppression of fever and parasites; and Decourt and his co-workers found that it had little effect upon natural infections of that species. According to Sinton, Hutton & Shute (1938) it had no true causal prophylactic action upon infections of the Roumanian strain of *P. falciparum* induced by sporozoites.

In its gametocidal action upon *P. falciparum* certuna resembles plasmoquine, but it appears to be in some cases less effective than that compound and to have no advantages, except lack of toxicity, to recommend its use in place of plasmoquine.

11. A COMPARISON OF THE RESPONSE TO THERAPEUTIC TREATMENT OF DIFFERENT SPECIES, OR OF STRAINS WITHIN SPECIES, OF AVIAN MALARIA

As in the study of the action of drugs upon avian malaria different strains, or even different species, have been used, it is essential that the action of compounds upon different species and strains should be compared under similar conditions, in order to discover whether differences in chemotherapeutic response can be detected between them.

Manwell (1930, 1932) studied the reaction of *P. cathemerium*, *P. elongatum*, three strains of *P. relictum*, and *P. rouxi* to plasmoquine and quinine. *P. elongatum* differs from the other species in that schizogony occurs chiefly in the bone marrow. *P. rouxi* (Sergent *et al.* 1929*a*) produces only four merozoites. Of the three strains of *P. relictum* one, called the type species, had been regarded by Hartman (1927) as a new species (*P. inconstans*), another was the Whitmore strain, and the third a German strain. With the exception of *P. rouxi*, for which intravenous injection was used as it cut down the incubation

Table 1. *Comparison of specific characters of* Plasmodium relictum, P. cathemerium, P. elongatum, P. circumflexum, P. rouxi *and* P. vaughani (*mainly after Manwell*, 1935)

	P. relictum = *P. praecox*	*P. cathemerium*	*P. elongatum*
Shape of gametocyte	Approximately spheroidal	Approximately spheroidal	Elongated
Shape of schizont (see Giovannola, 1939)	'Large and spheroidal. Displaces the nucleus of the parasitized red blood corpuscle'	'Large and spheroidal. Displaces the nucleus of the parasitized red blood corpuscle'	'Spheroidal, small, displaces the nucleus of the red blood corpuscle'
No. of merozoites (see Manwell, 1935)	8–15, 16–32 (according to strain)	6–24	8–12
Length of asexual cycle (Hewitt, 1940)	Whitmore strain 30 hr. Inconstans strain 36 hr. French strain 12 hr. Woodthrush strain 24 hr.	24 hr.	24 hr.
Character of pigment (see Manwell, 1935)	'Fine and dustlike in gametocytes'	'Coarse and often rodlike, especially in gametocytes'	'Clumped in asexual forms; scattered in gametocytes; granules round'
Type host (see Manwell, 1935)	English sparrow (=*Passer domesticus*) Spanish sparrow (=*Passer hispaniolensis*)	English sparrow (=*Passer domesticus*)	Canary (=*Serinus canarius*) English sparrow (=*Passer domesticus*)
Type of infection in canary (see Manwell, 1935)	'Acute stage longer than in *P. cathemerium* and *P. circumflexum*; parasite level in chronic stage higher'	'Acute stage usually short: parasite level very low in chronic stage'	Anaemia often very marked. Gametocytes only may be numerous in peripheral blood, as schizogony usually occurs in the bone marrow
	P. circumflexum	*P. rouxi*	*P. vaughani*
Shape of gametocyte	Elongated. End often curved round nucleus of red blood corpuscle	Elongated. Usually small	Elongated
Shape of schizont (see Giovannola, 1939)	'Elongated, does not displace the nucleus of the red blood corpuscle but tends to encircle it'	'Quadrangular or spheroidal, small. Never displaces the nucleus of the red blood corpuscle'	'Quadrangular, or spheroidal, small. Never displaces the nucleus of the red blood corpuscle'
No. of merozoites (see Manwell, 1935)	13–30	4	4–8, usually 4
Length of asexual cycle (Hewitt, 1940)	48 hr.	24 hr.	—
Character of pigment (see Manwell, 1935)	'Pigment scattered diffusely; single granules relatively small'	'Two unequal granules in asexual forms'	'One, two or occasionally three granules in asexual forms; often very refractile'
Type host (see Manwell, 1935)	Juniper thrush (=*Turdus pilaris*)	Algerian sparrow (=*Passer domesticus*) Canary (=*Serinus canarius*)	American robin (=*Turdus migratorius migratorius*)
Type of infection in canary (see Manwell, 1935)	'Acute stage usually short; parasite level very low in chronic stage'	'Acute stage long, and subsequent parasite level very high'	'Acute stage long (several weeks); parasite level often rather high during chronic stage'

period from 2–3 weeks to 4–5 days, the infections were transmitted from bird to bird by intramuscular injection of infected blood.

According to Manwell (1930) the three strains of *P. relictum* reacted to treatment with plasmoquine or quinine in essentially the same way, but marked differences were observed among the different species. Quinine and plasmoquine (Manwell, 1932) were almost equally effective upon *P. rouxi*. All birds infected with this species and treated during the incubation period with either drug were sterilized of their infections, and, even when treatment was delayed until the acute period, five out of seven birds were sterilized of the infection by quinine and at least nine out of eleven by plasmoquine. Upon the remaining three species plasmoquine was uniformly more effective than quinine. Of these three species *P. elongatum* was the most susceptible to plasmoquine and *P. cathemerium* the least. *P. cathemerium* and *P. relictum*, the two species which resembled each other most closely morphologically, differed least in their response to drug treatment. Infections of *P. cathemerium* were never sterilized either by plasmoquine or quinine alone (Manwell, 1930) or combined (Manwell, 1932). Treatment during the incubation period was more effective than during the acute stage. Only one out of nineteen birds infected with *P. relictum* was sterilized by curative treatment with plasmoquine, whereas six out of seven were sterilized by prophylactic treatment with the drug.

Plasmoquine was greatly superior to quinine in the treatment of infections of *P. elongatum* (Manwell, 1930), for all birds treated with plasmoquine, whether prophylactically or curatively, were sterilized, whereas only one out of twelve birds was sterilized by quinine, and this was after prophylactic treatment. A longer period of curative than of prophylactic treatment with plasmoquine was required to sterilize birds of infections of *P. elongatum* (Manwell, 1932).

Treatment with plasmoquine and quinine simultaneously was in no way superior to treatment with either drug alone. Except for its action upon *P. elongatum* plasmoquine was not, in Manwell's opinion, as superior to quinine as Roehl maintained.

There was no evidence in any of the four species of *Plasmodium* that the gametocytes were less suceptible to the drugs than the schizonts. This was contrary to the observations of Kopanaris (p. 8) who found the gametocytes more resistant to quinine than the schizonts.

Manwell's experience confirmed the view of Fourneau and his collaborators (1931) that increasing the dose of quinine or plasmoquine to the point of toxicity does not add to their efficacy.

Kikuth (1931) studied the action of plasmoquine and quinine upon *P. cathemerium*, *P. elongatum* and *P. inconstans* (=*P. relictum*). Using Roehl's method the difference in response to the drugs between the three species was very slight, but Kikuth did not state what proportion, if any, of the infections was sterilized. As the most striking difference, according to Manwell's results, in the therapeutic response of these three species was in the frequency with which the infections were or were not sterilized, it is unfortunate that Kikuth

gives no data on this point. Manwell (1934) considered that the six daily treatments of Roehl's method were probably insufficient to demonstrate specific differences in therapeutic response, and that this might explain why Kikuth found only an insignificant difference in the effect of plasmoquine and quinine upon *P. elongatum* as compared with *P. relictum*. Manwell stressed the need for experiments of this nature to be based upon a large number of birds as natural resistance differs in individual birds.

Manwell (1934) found *P. circumflexum* more resistant to quinine and plasmoquine than the species which he had studied previously.

The specific differences in response to drugs, particularly to plasmoquine, might, according to Manwell (1934), be correlated with specific differences in the rate of reproduction. That *P. rouxi* and *P. elongatum* were the two species most susceptible to plasmoquine was explained on this hypothesis by their slow rate of reproduction, which was assumed from the small number of merozoites formed, being 4 in *P. rouxi* and 8–12 in *P. elongatum*. *P. cathemerium*, which was the least susceptible to the drugs, on the other hand, reproduced most rapidly of all the species considered, producing an average of 15·4–15·5 merozoites every 24 hr.

Manwell believed that a similar correlation between the reproductive rate and susceptibility to plasmoquine might be traced in the three species of malaria of man, *P. falciparum* which produces 8–32 merozoites every 24–48 hr. being the least susceptible. This explanation fails to throw any light upon the high sensitivity of the gametocytes of this species to plasmoquine.

Atebrin neither prevented nor cured infections of *P. circumflexum* or *P. cathemerium* transmitted to canaries by blood inoculation (Manwell, 1933), but it sterilized canaries infected with *P. rouxi* and *P. vaughani* (Manwell & Haring, 1938). The ease with which atebrin sterilized infections of *P. vaughani* and *P. rouxi* contrasts with its action upon *P. relictum*, for both Kikuth and Tate & Vincent were unable to sterilize infections of this species with atebrin. In its high degree of susceptibility to plasmoquine and its low division rate *P. vaughani* resembles *P. rouxi*, and Manwell considered that this species furnished support to his hypothesis that the 'effectiveness of an antimalarial drug may bear an inverse relationship to the rate of reproduction of the parasite'.

The similarity in reaction to plasmoquine and quinine observed by Manwell (1930) in the three strains of *P. relictum* has not been confirmed by Lourie (1934*b*), who compared the effect of quinine administered under identical conditions to two of the three strains studied by Manwell. Both strains were transmitted to canaries by blood inoculation, and no morphological difference could be detected between them. Quinine hydrochloride was given daily by intraperitoneal injection, beginning with the day of inoculation. This daily quinine treatment held the Whitmore strain in check almost completely, whereas it was ineffective upon the German strain. The effectiveness of quinine upon the Whitmore strain as compared with the German strain was not correlated with a milder course of infection in the former strain than in the latter.

Lourie asserted that 'The practice of arranging various species of bird malaria in a sequence, in order of susceptibility to drugs' (Manwell, 1930, 1934), 'is no longer permissible, for there may be a greater difference in response to a drug between two varieties of the same species than between two strains of admittedly different species'.

The results of the work of different authors can be seen from the above brief account to be contradictory in many cases; but the methods used are not uniform, and as the criteria by which therapeutic response was assessed differed the results are not strictly comparable.

12. DRUG RESISTANCE IN AVIAN MALARIA

References to drug resistance in avian malaria are few. The Sergents (Et. & Ed. Sergent, 1921*a*, *b*) recorded the multiplication of parasites in spite of continued quinine treatment in a canary in which an infection of *Plasmodium relictum* had been held in check by the drug for 9 months. In the early account they stated that the parasites retained their resistance to quinine during two subsequent passages and lost it in the third. The effect of quinine upon the first two passages is not given in the later, detailed account, but in the third passage the infection was stated to be checked by quinine, though less readily than normally. They also described attenuation of virulence of *P. relictum* in six canaries as the result of quininization during 1–6 months, and found that it was restored after one, two or three passages. It is difficult to understand how virulence which is restored in the first passage can be assumed to have been lost when the criterion of loss of virulence is the intensity of infection in the first few passages made from the quininized bird. In the detailed account the data given indicate that in only three birds was subinoculation followed by feeble infections, and slight infections were produced in two out of nine birds inoculated from chronic, untreated control birds. In my own experience the infections obtained by subinoculation from chronic birds, which have never been treated with drugs, are frequently slight and do not attain to normal intensity for several passages, though considerable variation is found amongst individual birds.

Kritschewski & Rubinstein (1932) compared the incubation period in *Acanthis linaria* of two strains of *Plasmodium relictum* treated for 6 successive days with quinine, one of these strains having received four to six similar courses of quinine treatment previously in as many successive passages, whereas the other had received no such treatment. In 78·3 % of the birds infected with the previously quininized strain parasites appeared in the peripheral blood either on the day after treatment had ceased or within 4 days of this, whereas during the same period parasites were not seen in birds infected with the strain previously untreated. Kritschewski & Halperin (1933) found that sometimes, though not invariably, this quinine resistance persisted after the strain had passed through a developmental cycle in *Culex pipiens*.

Lourie (1935) subjected *Plasmodium cathemerium* in canaries to prolonged treatment with quinine, for 63, 100, or 137 days. The response of this quininized strain to further treatment with quinine, after inoculation into fresh birds, was studied and no evidence of drug resistance was observed. To avoid a possible diminution in the absorption of the drug by the erythrocytes, or its more rapid destruction by the tissues, due to the habituation of the birds to the drug after prolonged treatment, with the result that the amount of drug to which the parasites would be subjected would become gradually reduced, Lourie passaged the strain through canaries at intervals of 2 or 3 weeks, at the same time subjecting each bird daily to treatment with quinine. The strain was thus exposed to uninterrupted quinine treatment for a maximum of 511 days, but it did not lose its sensitivity to the drug. The experiments were repeated with plasmoquine and gave similar results.

Attenuation of virulence did not result from prolonged treatment either with quinine or with plasmoquine.

Lourie's strain of *P. cathemerium* was subjected to a much longer treatment with quinine than Kritschewski & Rubinstein's strain of *P. relictum*, but no loss of sensitivity resulted. The results of these workers therefore appear contradictory, and as Lourie pointed out, both can be accepted only if further work shows that different species or strains of malaria differ in their readiness to acquire drug resistance.

13. The influence of the reticulo-endothelial system upon the therapeutic action of antimalarial compounds

Kritschewski & Meerson (1932) found that in birds (*Acanthis linaria* and *Spinus spinus*) in which the reticulo-endothelial system was blocked by trypan blue and which subsequently were inoculated with *Plasmodium relictum*, the incubation period was shorter than in birds in which the reticulo-endothelial system was normal. They concluded that in the bird this system was an important defence mechanism against malarial infection. Causey (1939) observed no difference in the course of infection in splenectomized and non-splenectomized birds infected with *P. cathemerium* or *P. relictum* when inoculated with blood from the same species of host; but when the strain of parasite introduced was from a host of a different species the spleen was of importance in defence, and he concluded that it prevented acute infection by the removal and destruction of the foreign erythrocytes and incidentally of the parasites.

According to Kritschewski & Demidowa (1935), when birds (*Acanthis linaria*) were infected with *Plasmodium relictum* and treated with six successive daily doses of plasmoquine, quinine or acrichin 8 (p. 40), parasites appeared more rapidly in those in which the reticulo-endothelial system had been blocked with trypan blue than in normal birds. They concluded that the blocking of the reticulo-endothelial system reduced, in a great measure, the efficacy of the drugs.

14. THE EFFECT OF DRUGS UPON SPOROZOITE-INDUCED INFECTIONS OF AVIAN MALARIA

In their work upon induced malaria in the treatment of general paralysis of the insane Yorke & Macfie (1924) found that whereas quinine given prophylactically was remarkably effective as a preventive against malaria transmitted by blood inoculation it was useless, unless administered for 10–14 days, against mosquito-induced malaria. They concluded that quinine had little, if any, action upon the sporozoites themselves, and that the development of infection was prevented by the curative action of the drug upon the trophozoites which developed from them.

A remarkable difference in response to prophylactic treatment with certain antimalarial compounds has also been observed in infections of avian malaria induced by sporozoites as compared with those induced by blood inoculation. Russell & Nono (1932) found that neither plasmoquine nor chinoplasmin, in the maximum tolerated dose, had any prophylactic action upon infections induced by sporozoites whether introduced by the bite of *Culex quinquefasciatus* (=*fatigans*) or by syringe.

Tate & Vincent (1933*a*) found that plasmoquine given prophylactically, even in doses as small as 0·02 mg. per 20 g. of bird, caused great delay in the appearance of *Plasmodium relictum* in the peripheral blood of canaries infected by blood inoculation, but in mosquito-infected birds doses of 0·32 mg. had no effect. In mosquito-induced infections parasites did not appear in the blood during treatment, but they appeared soon after its termination.

Kikuth & Giovannola (1933) studied the effect of quinine, plasmoquine and atebrin, given prophylactically, upon infections of *P. cathemerium* and *P. relictum* transmitted by the injection of a suspension of sporozoites. Some birds proved refractory to the infection, a condition never encountered by Kikuth in thousands of birds infected by blood inoculation. Whereas in the birds treated with quinine or plasmoquine there was no delay in the appearance of parasites in the peripheral blood as compared with the controls, in those treated with atebrin the appearance of parasites was delayed and the resulting infection was very slight. The authors concluded that neither quinine, plasmoquine, nor atebrin had any true causal prophylactic action, but that atebrin could be regarded as a clinical prophylactic. They attributed the delaying action of atebrin upon the parasites to its slow excretion from the body, a characteristic which had been demonstrated by several workers.

Tate & Vincent (1934*b*) found no evidence of such a clinical prophylactic action of atebrin upon infections of *P. relictum* transmitted to canaries by the bite of *Culex pipiens*. These authors agreed with Kikuth & Giovannola that atebrin might diminish the severity of the attack, but its onset was not retarded. The action of atebrin was, in their experience, similar to that of plasmoquine—highly effective against infections transmitted by blood inoculation but ineffective against mosquito-transmitted infections. In mosquito-

borne infections, according to Tate & Vincent, atebrin was, in one respect, inferior to plasmoquine, for when treatment with atebrin was continued beyond the normal period of incubation parasites appeared in the blood before the end of treatment, whereas with plasmoquine their appearance was delayed until several days after treatment had ceased.

Russell (1934) found that atebrin, in the maximum tolerated dose, did not prevent the appearance of parasites in the peripheral blood of canaries infected with sporozoites of *P. capistrani*,[1] though it did appear to reduce the mortality rate of the birds.

15. The exo-erythrocytic cycle of development in avian malaria

The cause of the difference in response to drugs of sporozoite-induced infections from those induced by blood inoculation is one of the most important problems in the therapeutics of malaria. Its solution may hinge upon the fate of the sporozoites in the tissues of the vertebrate host. The direct penetration of the sporozoites into the red blood corpuscles described by Schaudinn (1903) has never been confirmed. James (1931) revived and amplified a suggestion made by Grassi (1899) that the sporozoites on entry into the vertebrate host undergo a cycle of development before passing into the red blood corpuscles. This cycle, James suggested, might take place in the reticulo-endothelial cells of the internal organs, as in the case of *Haemoproteus*.

Within the last few years a cycle of development in the internal organs, in cells other than the erythrocytes, has been discovered in several species of malaria, and the relationship of these non-pigmented forms to the sporozoites has been the subject of discussion, which has been reviewed by Porter & Huff (1940).

It was noted by Huff (1930) that in *Plasmodium elongatum* schizogony was rare in the peripheral blood but abundant in the haemopoietic tissues. With Bloom (Huff & Bloom, 1935) he observed that schizogony took place in the erythroblastic cells of the bone marrow as well as in the erythrocytes. In most of these cells the parasites were probably ultimately phagocytized, but those occasionally seen in the non-phagocytic plasma cells presumably survived. Raffaele (1934, 1936*a*) suggested that there was a double cycle of development in *P. elongatum*, one type of schizogony taking place in the erythrocytes and the other in the reticulo-endothelial cells of the internal organs, particularly in the liver, spleen and bone marrow. He (Raffaele, 1936*b*) also found non-pigmented schizogonic stages, though few in number, in the internal organs of canaries infected with sporozoites of *P. relictum*. James & Tate (1937*a*, *b*, 1938) found a schizogonic cycle in the reticulo-endothelial cells of the internal organs of fowls infected with *P. gallinaceum*, in addition to the schizogonic cycle in the erythrocytes. As these non-pigmented schizonts infect circulating leucocytes, especially monocytes, in addition to cells of the reticulo-endothelial

[1] Manwell (1936) is of the opinion that *Plasmodium capistrani* and *P. relictum* are synonyms.

system, James & Tate proposed the term *exo-erythrocytic* to distinguish them from forms developing in the erythrocytes. James & Tate described and figured exo-erythrocytic forms of *P. gallinaceum* varying in size from small oval bodies with a single mass of chromatin, to large schizonts containing many chromatin masses, and the subsequent formation of many merozoites.

Exo-erythrocytic schizogonic forms were found by Kikuth (1937) in canaries infected with *P. cathemerium*, and by Manwell & Goldstein (1939) in *P. circumflexum*. The development of exo-erythrocytic schizonts from uni-nucleate, non-pigmented forms to multinucleate forms was observed by Kikuth & Mudrow (1939*b*) in the tissues at the site of inoculation in canaries infected with sporozoites of *P. cathemerium*; but although uni-nucleate stages were found 16 hr. after inoculation, and therefore too soon to have been derived from erythrocytic forms since the duration of the schizogonic cycle of this species is 24 hr., the actual transformation of sporozoites into uni-nucleate exo-erythrocytic forms was not observed.

The question of the relationship of the exo-erythrocytic schizonts to the sporozoites is complicated by the fact that these forms are present in infections induced by blood inoculation (Huff & Bloom, 1935; Brumpt, 1937; Kikuth, 1937; James & Tate, 1938; Rodhain, 1938; Corradetti, 1938; Manwell & Goldstein, 1939), as well as in those induced by sporozoites.

Experiments with *P. gallinaceum* in fowls led James (1939) to conclude that in addition to developing from sporozoites the exo-erythrocytic schizonts can arise from erythrocytic forms. Coulston & Manwell (1940) confirmed this view by infecting birds with single red blood corpuscles containing *P. circumflexum* and obtaining infections which in the early passages were very slight and devoid of exo-erythrocytic schizonts, but which in later passages exhibited these forms.

Whether the development of exo-erythrocytic, tissue-inhabiting parasites from erythrocytic forms has any bearing upon the phenomenon of relapse has not been established.

Kikuth & Mudrow (1937, 1938) believed that the number of exo-erythrocytic forms and the severity of the disease were correlated, and that the difference in virulence between the different species and strains of avian malaria might be explained, at least in part, by the presence or absence of these forms: thus infections of *P. relictum* in which exo-erythrocytic schizonts were scanty and soon disappeared were less virulent than those of *P. cathemerium* in which they were numerous and persisted longer.

Although direct evidence of the development of sporozoites into exo-erythrocytic schizonts has not yet been obtained the occurrence of such forms in several species of avian malaria is beyond dispute. As their environment in the host is different from that of the erythrocytic forms their response to antimalarial compounds is of interest even if their relationship to the sporozoites should not be proved.

James & Tate (1937*b*, 1938) discovered that chickens in which quinine had

caused a temporary disappearance of parasites from the peripheral blood, died later, and post-mortem examination revealed large schizonts in the cells lining the capillaries of the brain, sometimes occluding the lumen. Raffaele (1938) found numerous exo-erythrocytic forms of *P. gallinaceum* in the internal organs of infected fowls which had been treated with atebrin, whereas in the peripheral blood only a very few young trophozoites could be seen.

Kikuth & Mudrow (1939*a*) compared the prophylactic action of quinine, atebrin, certuna and plasmoquine upon infections of *P. cathemerium* in canaries, and found the exo-erythrocytic parasites to be more resistant to drugs than the erythrocytic forms, and that a fresh supply of erythrocytic parasites might be produced continually from them to take the place of those destroyed by the drug.

16. Chemical constitution and antimalarial activity

A. *Method of assessing the activity of a compound*

Avian malaria has contributed to our knowledge of the relative antimalarial activity of the natural cinchona alkaloids, and of the relationship between therapeutic activity and chemical constitution in these and in new synthetic compounds. The technique used and the criteria by which antimalarial activity has been assessed have varied. In all these experiments the infections have been transmitted by blood inoculation; but Goodson, Henry & Macfie (1930) used the intraperitoneal method which may account for the wide variation (3–11 days over a period of 2 years) in the incubation period amongst the controls, for Giemsa (1933) found the variation to be greater after this method than after intramuscular injection.

In testing the antimalarial properties of compounds synthesized by Magidson and his collaborators, Kritschewski & Sternberg (1933) used *Acanthis linaria* and *Spinus spinus* in place of canaries. In these birds the course of infection with *Plasmodium relictum* was very virulent and usually ended fatally.

The drugs were injected subcutaneously (Giemsa, Weise & Tropp, 1926), intramuscularly (Kritschewski & Sternberg, 1933), or orally (Goodson *et al.* 1930; Buttle, Henry & Trevan, 1934; Buttle, Henry, Solomon, Trevan & Gibbs, 1938; Tate & Vincent, 1933*a*). Whereas some workers have studied the curative action of the drugs others have studied their prophylactic action.

Kritschewski & Sternberg defined their curative dose as the smallest amount which in six daily doses would prolong the incubation period for 2–3 days beyond that of the untreated birds. From my own experience such a short period of delay is not significant unless it is constant over a very large series, as it is not beyond the normal variability within the incubation period.

Buttle and his collaborators (1934) compared the antimalarial activity of the four primary cinchona alkaloids and their dihydro derivatives in terms of the dose of quinine equal in antimalarial efficacy to unit doses of these com-

pounds. Activity was also estimated by comparing the average time of appearance of parasites in six birds treated with a drug with that of a similar number treated with quinine.

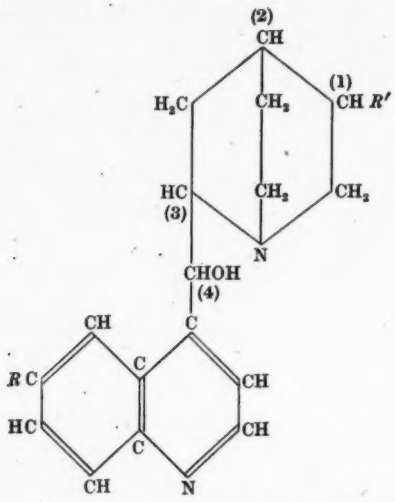
Buttle and his collaborators (1938) also employed quinine as a standard in comparative tests of antimalarial activity. Constant doses of 2·5 mg. of quinine were given, by Roehl's method, to one group of four to six birds and of 2·5 mg. of the drug to be assessed to a similar group. The average time of incubation was noted in each group and also in a group of untreated controls. The activity of the compounds was compared in terms of the *quinine ratio.* This was obtained by dividing the difference between the average time of incubation in the group receiving the drug and in the controls, by the difference between the average time of incubation in the group receiving quinine and in the controls. When drugs were more active than quinine and therefore their ratio was greater than 1 no estimate of their relative activity could be given, except that drugs which produced a longer incubation period than quinine were more active. Since for each group tested one group of birds was treated with quinine, the average time of appearance of parasites was assessed for as many groups of quininized birds as there were drugs tested. If the average time of appearance of parasites is compared throughout the quininized groups a wide divergence will be found. Inasmuch as each of the drugs other than quinine was tested upon only one group of birds it seems justifiable to ask whether a variability as great as that amongst the quininized groups might not have been found for any one drug if it had been tested over as wide a series as the quinine series; and whether if such variability did occur the variation in both the drug and corresponding quinine group would always have been in the same direction. If not the quinine ratio would have been affected by the variation.

Fourneau and his co-workers (Fourneau *et al.* 1931, 1933) used infections of *Haemoproteus* in the rice finch, *Padda orizivorae,* for assessing the antimalarial activity of the compounds which they had synthesized. They considered a drug to be effective if it caused a disappearance of the parasites from the peripheral blood of a group of three birds for at least 2 days. It will be remembered that in *Haemoproteus* (p. 17) only gametocytes are present in the peripheral blood. Fourneau and his collaborators use the term 'sterilized' for this temporary disappearance of gametocytes. It is obvious that sterilization in this sense is not comparable with sterilization as applied to infections of *Plasmodium* in canaries in which case it means complete eradication of all parasites.

B. *The cinchona alkaloids and their derivatives*

Giemsa and his collaborators (1926) found that the action of some cinchona alkaloids and their derivatives known to be active against malaria of man had a strikingly similar action against *Plasmodium relictum* in canaries. Thus quinethylene, which according to Giemsa & Werner (1914) had a beneficial

action upon human malaria, was active against avian malaria. Quinpropylene, which was less effective against malaria of man, and quinamylene of which the action against human malaria was not known, were both active against avian malaria.



General formula of cinchona alkaloids (after Goodson, Henry & Macfie 1930).

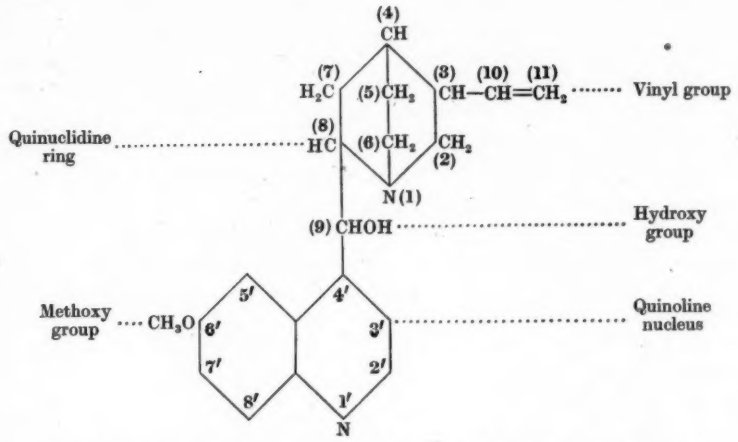
'R = H in cinchonine, cinchotenine, dihydrocinchonine, cinchonidine, cinchotenidine and dihydrocinchonidine: in all other cases it is —OCH_3, except in cupreine where it is —OH and the cupreine alkyl ethers, quinethyline (—OC_2H_5); quinpropyline (—OC_3H_7); quinamyline (—OC_5H_{11}).

'R' = —$CH{:}CH_2$ in cinchonine, cinchonidine, quinine, and quinidine.
= —CH_2CH_3 in the dihydro-reduction products of these four alkaloids.
= COOAlk. for the alkyl-quitenines, quitenidines, cinchotenines and cinchotenidines; Alk. = methyl, ethyl, propyl, etc.

'Carbon atoms 1, 2, 3, 4 are all dextrorotatory in cinchonine and its derivatives and in quinidine and its derivatives: carbon atoms 1 and 2 are dextrorotatory and 3 and 4 laevorotatory in cinchonidine and quinine and their derivatives.' (Goodson, Henry & Macfie, 1930.)

Giemsa and his collaborators (1926) studied the action upon avian malaria of a series of compounds which varied slightly in chemical constitution, in order to try to discover what relationship there might be between therapeutic activity and chemical structure. The hydrogenation of the vinyl group of quinine to form hydroquinine ($>$CH—CH_2—CH_3) produced little change in activity, hydroquinine being slightly less active than quinine. The hydrogenation of cupreine into hydrocupreine changed an almost inactive compound into one which delayed the appearance of parasites 10–14 days beyond the controls, though later Giemsa & Oesterlin (1933) failed to find such a great increase in activity. Addition in the vinyl group to form hydrochlorquinine ($>$CH—CHCl—CH_3), hydroiodoquinine ($>$CH—CHI—CH_3) and

quinine dibromide (>CH—CHBr—CH_2Br) caused only a slight loss in activity. In dehydroquinine (>CH—C≡CH) antimalarial activity was reduced. In quitenine (>CH—COOH), in which the vinyl group is oxidized to carboxyl, antimalarial activity disappeared completely, but in the ethyl ester it was restored.



Quinine. Numbering according to Rabe (*Ber. dtsch. chem. Ges.* 1922, **55**, 522).

Variations in the central —CHOH— group were found by Giemsa and his collaborators to be accompanied by loss of activity as in quinine chloride (—CHCl—), quinicine (p. 35), quininone (—CO—), desoxyquinine (—CH_2—), quininine (: CH—), and acetylhydroquinine (—CHOAc—).

Substitutions in the 5′ position in the quinoline nucleus of hydroquinine to form 5-aminohydroquinine, 5-chlorhydroquinine, and 5-bromohydroquinine produced no essential change in activity.

Azo dyes synthesized from hydroquinine were, according to Giemsa & Oesterlin (1933), almost always inactive against bird malaria; but C 77, 6-methoxyquinoline-8-azo-hydrocupreine, was an exception and had a chemotherapeutic index of 1 : 8. This compound was stated by Missiroli & Mosna (1934) to be effective in cases of malignant tertian malaria.

Goodson and his collaborators (1930) found hydroquinine to be the most active of the natural cinchona alkaloids against avian malaria, followed by quinidine, quinine, cinchonidine, and cinchonine in descending order of activity, though there was little difference between the last four. These results are not in complete agreement with those of Giemsa and his collaborators, who found hydroquinine slightly less active than quinine, and cinchonine definitely less active than quinine, quinidine and hydroquinine. The technique used in these two sets of experiments was not identical (p. 30) and the difference in

method may account for the difference in results, for Giemsa (1933) was of the opinion that when given orally quinine and hydroquinine would be absorbed and eliminated in the same way, whereas when injected subcutaneously the less soluble quinine would be eliminated more slowly and remain active longer than the more soluble hydroquinine. Giemsa & Oesterlin (1933) found hydroquinine more active when given orally than when given subcutaneously.

Goodson and his collaborators found that whereas the hydrogenation of quinine produced the more active hydroquinine, the hydrogenation of quinidine, cinchonidine, and cinchonine did not increase their antimalarial activity. Buttle and his collaborators (1934) also found the effect of hydrogenation different for different alkaloids, for whilst it increased the effect of quinine and possibly quinidine it probably lessened the effect of cinchonine. Hydroquinidine, cinchonidine and quinidine were definitely less active than quinine, quinidine being less active than Goodson and his collaborators had found it to be, and cinchonine the least active of these alkaloids.

Goodson and his collaborators confirmed the observation of Giemsa and his collaborators that whereas quitenine was inactive its ethyl ester was active. They found that, on the whole, antimalarial activity increased as the homologous series of esters was ascended, reaching a maximum at butyl- or amyl-quitenine, at which point infections were sterilized in two birds. But apart from these two sporadic cures the activity of these esters did not appear to approach that of hydroquinine or even quinine. Buttle and his collaborators (1934) failed to find such a marked rise in activity from ethyl- to amylquitenine. They suggested that the variability in results might be due to the ease with which the alkylquitenines were hydrolysed and to a possible difference amongst canaries in capacity for hydrolysing the drugs.

Whereas methylquitenine dihydrochloride showed slight antimalarial activity (Goodson *et al.* 1930; Cohen & King, 1938) methylcinchotenine and methylcinchotenidine dihydrochlorides were both inactive (Cohen & King, 1938), though according to Goodson and his collaborators the ethyl esters were slightly active. Quitenamide and quitenmethylamide were both without therapeutic action.

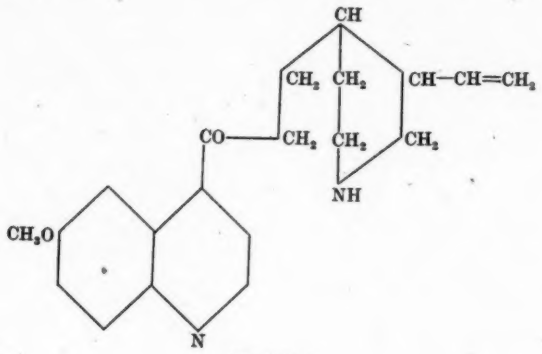
The loss of antimalarial activity noted by Giemsa and his collaborators when the central —CHOH— group of quinine was replaced by —CHCl— to form quinine chloride was confirmed by Buttle and his collaborators (1934), who also found that dihydroquinine chloride and cinchonidine chloride were likewise inactive. Cohen & King (1938) confirmed and amplified these observations, finding that quinine chloride, hydroquinine chloride, quinidine chloride, hydroquinidine chloride, cinchonine chloride, cinchonidine chloride and hydrocinchonidine chloride were all inactive, as also were quinene (= quininine) and the analogous derivatives of cinchonine and hydroquinine.

The inactivity of desoxyquinine (—CH_2—) discovered by Giemsa and his collaborators was found by Cohen & King to be shared by desoxycinchonine,

desoxycinchonidine, and desoxydihydrocinchonine and desoxydihydrocinchonidine.

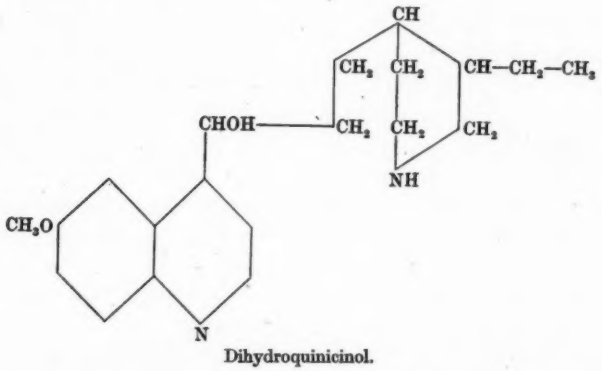
These observations therefore show that alterations in the central —CHOH— group lead to the loss of antimalarial activity.

Whereas in quinine the presence of the —CHOH— group appears to be essential to antimalarial activity Ainley & King (1938) found that the reduction to the carbinol of quinicine, an isomeride of quinine shown by Giemsa and his collaborators and by Giemsa & Oesterlin to be inactive, did not restore



Quinicine.

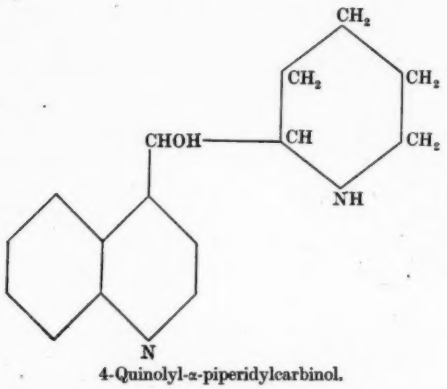
the activity, though the dihydroquinicinols, which occurred in two diastereoisomeric forms, bore so close a resemblance to quinine. A methylation product, *d*-*N*-methyldihydroquinicinol, also proved inactive.



Dihydroquinicinol.

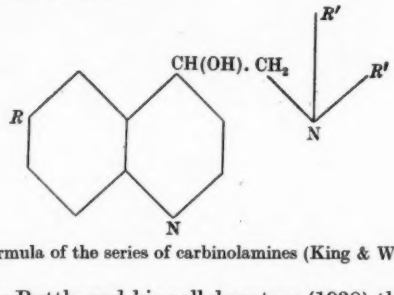
Retaining the central carbinol group Ainley & King then synthesized 4-quinolyl-α-piperidylcarbinol, 4-(6-methoxyquinolyl)-α-piperidylcarbinol and their alkyl derivatives. No quinolyl derivative without the 6-methoxy group showed any antimalarial activity. 4-(6-methoxyquinolyl)-α-piperidylcarbinol

had approximately the same M.T.D. as quinine but was only about half as active as that drug.



4-Quinolyl-α-piperidylcarbinol.

Continuing the search for antimalarial compounds built on the pattern of the cinchona alkaloid molecule but of simpler structure King & Work (1940) synthesized a series of simple carbinolamines 'in which the strongly basic nitrogen centre was still separated from the quinoline nucleus by two carbon atoms as in quinine and hydroquinine'. In the methoxyquinolyl series ($R = OMe$) active compounds were obtained where $R' = C_4H_9$, C_5H_{11} or C_6H_{13}. Higher or lower homologues were inactive.



General formula of the series of carbinolamines (King & Work, 1940).

It was shown by Buttle and his collaborators (1938) that epimerization at C^9 reduced the activity of quinine, in terms of the quinine ratio (p. 31), from 1 to 0·1 in *epi*-C^9-quinine, and of quinidine from 0·5 to 0·1 in *epi*-C^9-quinidine. Whilst the quinine ratio of dihydroquinine was 1·35, that of *epi*-C^3-dihydroquinine was 1·0. But no obvious relationship was traceable between optical and antimalarial activity in the cinchona alkaloids. Thus while the *l*-bases, quinine and cinchonidine, were more active than the *d*-bases, quinidine and cinchonine (Buttle *et al.* 1934), *apo*quinidine methyl ether is more active than either of its *l*-isomerides, α- or β-isoquinine.

As Buttle, Henry & Trevan (1934) had suggested that irregularities in the antimalarial action of the alkylquitenines might be due to the ease with

which these compounds are hydrolysed, Buttle and his collaborators (Buttle *et al.* 1938) decided to investigate an homologous series of more stable derivatives of the cinchona alkaloids. They chose the alkyl ethers of *apo*quinine and dihydrocupreine. No regularity in rise and fall in antimalarial activity was observed in the homologous series of either substance, but if each of the two series was divided into two groups, one containing odd and the other even numbers of carbon atoms, there was, with certain exceptions, a tendency for activity to rise to a maximum and then fall as each of the four series was ascended. A rise in activity as the side chain was increased in length was also observed in a short series of dihydrocupreidine ethers. The authors, whilst pointing out that it is as yet impossible to draw any precise conclusions of the mode of antimalarial action of the cinchona alkaloids, believe their action to be directly parasiticidal, and conclude that alterations in structure which produce a weaker base (e.g. quinine to quitenine) reduce or destroy activity. 'That inactivity ensues from even a spatial distribution change as well as from small structural changes in the connecting —CHOH— group seems to imply that the essential condition for activity is a close "fit" between the drug and the protein molecules of the parasite....'

C. *Plasmoquine and related compounds*

In addition to the natural cinchona alkaloids and synthetic compounds closely related to them the antimalarial properties of compounds related to plasmoquine (p. 14) and atebrin (p. 18) have been studied. These compounds have been synthesized with the twofold objective of finding antimalarial drugs of greater efficacy than those already known, and of gleaning knowledge of the relationship of therapeutic activity to chemical constitution.

Fourneau and his collaborators (1931, 1933) prepared a series of compounds related to plasmoquine, the majority of which had a dialkylaminoalkylamino side chain attached at position 8 to the 6-methoxyquinoline nucleus (p. 14). Thirty-eight out of forty-eight of these compounds showed some activity when tested against infections of *Haemoproteus orizivorae* in the rice finch, and in seven the chemotherapeutic index was not less than 1/40, which was the index obtained for plasmoquine by this method. Fourneau 710, later known as rhodoquine, 6-methoxy-8-diethylamino-*n*-propylaminoquinoline (side chain ($NH—CH_2—CH_2—CH_2—N(C_2H_5)_2$), was less toxic than plasmoquine and had an index of 1/100 (Fourneau *et al.* 1933). The Sergents and co-workers (1931) found that it exerted a definite antimalarial action upon infections of *Plasmodium relictum* in canaries when given either curatively or prophylactically. When given curatively its therapeutic index was 10 times greater than that of quinine.

Fourneau 664, ($—NH—CH_2—C(CH_3)_2—CH_2—N(C_2H_5)_2$), was less toxic to rice finches than F 710 and had an index of 1/40. Both these substances were found by Monier (1931*a*, *b*) to be of value in the treatment of benign tertian malaria, and F 710 and the Russian product plasmocide, which are identical in structure, are used in the treatment of malaria. Many of the quinoline

compounds synthesized by Fourneau were unstable and became toxic, and so were unsuitable medicinally.

From the large series of 8-aminoquinoline compounds which they had synthesized, Fourneau and his collaborators (1931, 1933) made the following general deductions upon the relationship of chemical structure to antimalarial activity. Compounds in which the side chain, substituted at position 8 of the quinoline nucleus, was normal showed greater antimalarial activity than those in which it was branched; thus the index of F 735,

$$(\text{—NH—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—N(C}_2\text{H}_5)_2),$$

was 1/150, whereas that of plasmoquine,

$$(\text{—NH—CH(CH}_3\text{)—CH}_2\text{—CH}_2\text{—CH}_2\text{—N(C}_2\text{H}_5)_2),$$

was 1/40, and that of F 696,

$$(\text{—NH—CH(C}_2\text{H}_5\text{)—CH}_2\text{—CH}_2\text{—N(C}_2\text{H}_5)_2),$$

was 1/4. Though the presence of a methoxy group in position 6 of the quinoline nucleus favoured antimalarial activity it was not indispensable, for two compounds in which the 6-methoxy group was replaced by H and OH had an index of 1/80 and 1/40 respectively. The substitution of an ethoxy group lowered the index from 1/100 in F 710 to 1/4 in F 730 (6-ethoxy-8-diethylamino-propylaminoquinoline). The substitution, in a single compound, of a methyl group for the methoxy group at 6, was followed by complete loss of activity.

Magidson and his collaborators have also studied the relationship between chemical structure and antimalarial activity in many synthetic quinoline compounds. The results of some of the biological tests have been described by Kritschewski & Sternberg (1933, p. 30). As neither 8-amino-6-methoxyquinoline nor its mono- nor dialkyl- substitution products possesssed any antimalarial activity, Magidson & Strukow (1933*a*) turned their attention to compounds of the type $6\text{—}R\text{—}Q\text{—}8\text{—NH—CH}_2\text{—CH}_2\text{—N(C}_2\text{H}_5)_2$, where Q = quinoline nucleus and R = hydroxy, methoxy, ethoxy, propyloxy, *iso*propyloxy, butyloxy, *iso*butyloxy, *iso*amyloxy or octyloxy. The maximum therapeutic effect upon avian malaria was obtained with the methoxy compound, and it diminished with the increase in number of the C atoms in the 6-alkoxy side chain, until in the *iso*amyloxy group it had disappeared. Baldwin & Robinson (1934) stated that 6-methoxyquinolines usually showed greater antimalarial activity than 6-ethoxyquinolines, as Fourneau also had found.

In studying the influence of the length of the side chain in position 8 on the quinoline nucleus in a series $6\text{—CH}_3\text{O—}Q\text{—}8\text{—NH—(CH}_2\text{)}n\text{—N(C}_2\text{H}_5)_2$, where Q = quinoline nucleus and $n = 2, 3, 4$ or 5, Magidson & Strukow (1933*b*) found that the maximum antimalarial activity was reached when $n = 3$. This compound, similar in constitution to F 710 (rhodoquine), had a chemotherapeutic index of 1/26·5 when tested against *Plasmodium relictum*. The index of 1/100 obtained by Fourneau was the result of tests upon *Haemoproteus orizivorae*. Plasmocide (Kritschewski & Sternberg, 1933) is the methylene disalicylate

of this base. It is said to be less toxic than plasmoquine and to be efficacious clinically. The Russian workers claim that plasmocide is easier and cheaper to synthesize than plasmoquine.

In a further series where $n=6$, 7, 9 and 11 the corresponding chemotherapeutic indices were 1/13·3, 1/33·3, 1/40 and 1/5 (Magidson *et al.* 1935). From the results of the complete series of compounds it was found that homologues with an even number of carbon atoms in the side chain at 8 were less active therapeutically than those containing an uneven number. The series of compounds having an uneven number of carbon atoms had two maxima of antimalarial activity, the first being when $n=3$ with an index of 1/26·5 and the second when $n=9$ with an index of 1/40. The wide range of activity shown by this compound was due to its low toxicity. The antimalarial activity of compounds where $n=1$, 8 or 9 was not described.

The method used by Kritschewski & Sternberg (1933) and Magidson and his collaborators for testing antimalarial activity assessed schizontocidal action. Bovet and his collaborators (1934) compared the gametocidal action of a series of 6-methoxy-quinolines with a side chain at 8 in which the number of carbon atoms varied from 2 to 11. These tests were made upon *Haemoproteus* in the rice finch. The maximum antimalarial activity occurred when the number of carbon atoms was 5 or 6, at which point the chemotherapeutic index was 1/150. In higher homologues the index declined, and at C_{11} was reduced to 1/3.

Bovet & Demanche (1933) stressed that whereas compounds which were purely schizontocidal, e.g. quinine and atebrin, were without action upon *Haemoproteus*, both gametocidal and schizontocidal compounds show some degree of activity against *Plasmodium* infections in canaries. An increase in length of the side chain at 8 on the 6-methoxyquinoline nucleus was accompanied by a reduction in gametocidal action and an increase in schizontocidal action. When F 852, 8-diethylamino-undecylamino-6-methoxyquinoline ($6—CH_3O—Q—8—NH—(CH_2)_{11}—N(C_2H_5)_2$), was tested upon *Haemoproteus* in the rice finch the chemotherapeutic index was 1/3 as compared with 1/40 for plasmoquine, but upon infections of *Plasmodium* in canaries an index of 1/100 was obtained as compared with 1/30 for atebrin or plasmoquine, or 1/4 for quinine. F 852 therefore had a strong schizontocidal action, but was not devoid of gametocidal action. This combination of schizontocidal and gametocidal properties Bovet & Demanche termed *amphotropism*. According to Bovet and his collaborators (1934) schizontocidal compounds were formed either by an increase in the molecular weight of the nucleus as compared with plasmoquine, e.g. atebrin, or by the lengthening of the side chain, e.g. F 852.

Plasmoquine and nearly related compounds have anaesthetic properties which, on account of their toxic effect, are disadvantageous in antimalarial compounds. Bovet & Demanche found that in F 852 the anaesthetic action had disappeared almost completely, which accounted for the low toxicity of this compound as compared with compounds with shorter side chains. They pointed out that Fourneau & Samdahl in the piperazines, and Miescher in the

percaïnes, found that anaesthetic action, having reached a maximum, decreased with the further increase in length of the side chain.

Bovet & Demanche (1933) found that alterations in the chemical structure of F 852, similar to those made previously (Fourneau *et al.* 1933) in other 8-aminoquinolines, produced similar changes in chemotherapeutic activity; thus the substitution of an H group in place of the 6—CH_3O— group reduced the activity, whereas the substitution of a CH_3— group led to a complete loss of activity.

According to Magidson & Strukow (1933*b*) the interpolation of an OH group in the side chain at position 8 on the quinoline nucleus in plasmocide reduced the chemotherapeutic index from 1/26·5 to 1/14, this decrease in activity being due principally to increased toxicity. Branching in the side chain at 8 (Magidson *et al.* 1934) lowered the antimalarial activity, as Fourneau also had discovered. When an ethyl group was introduced into the amino group at 8, with the formation of a tertiary amine, antimalarial activity disappeared (Magidson & Strukow, 1933*b*). The replacement of the diethylamino group by a piperidino group lowered the index from 26·5 to 6 though the structure of the resulting compound approached that of quinine.

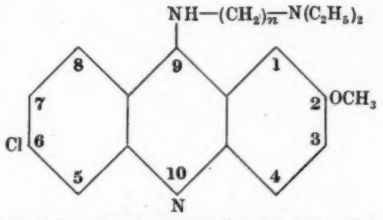
Compounds of the plasmoquine type, prepared by Sir Robert Robinson and his collaborators (Barger & Robinson, 1929), were tested for antimalarial activity by Tate & Vincent (1933*b*) upon *P. relictum* by Roehl's method (p. 13). Thirteen of the compounds showed antimalarial activity, and in two of them the index was 1/16. Their action resembled that of plasmoquine. R 44 (8-γ-aminopropylamino-6-*n*-butoxyquinoline), though its index was only 1/8, had a sterilizing power equal to or greater than that of plasmoquine. The authors found that in such quinoline compounds very slight changes in chemical constitution might cause great changes in antimalarial activity.

D. *Atebrin and related compounds*

Compounds related to atebrin have been synthesized by Magidson and his collaborators. Acrichin 5, which is the dihydrochloride of 2-methoxy-6-chlor-9-diethylamino-propylaminoacridine, had a chemotherapeutic index of 1/15 when tested upon *P. relictum*, and acrichin 8, the dihydrochloride of 2-methoxy-6-chlor-9-diethylamino-butylaminoacridine, had an index of 1/20 (Kritschewski *et al.* 1934).

In a series of acridine derivatives with a 9-dialkylamino-alkylamino side chain compounds in which there was no substitution at 6 or 7 on the acridine nucleus were inactive (Magidson & Grigorowsky, 1936); thus 2-methoxy-9-[(γ-diethylaminopropyl)amino]-acridine had an index of 0. When a nitro-group was introduced at 6 the compound was still inactive, but when it was moved to 7 the resulting compound showed slight activity. When a Cl atom was substituted at 6 a series of very active compounds was obtained (formula, p. 41, $n=2$, 3, 4 or 5). As in the series related to plasmoquine (p. 38) an increase in length of the side chain was accompanied by an increase in anti-

malarial activity, reaching a maximum index of 1/20 when $n=4$, after which activity declined. Branching in the side chain, studied in two compounds only, led to an increase in activity in one case and a decrease in the other. The interpolation of an hydroxy group in the side chain of the propyl derivative reduced the index.



General formula of compounds related to atebrin.

An increase in the molecular weight of the 2-alkoxy group led to a loss of activity due to increased toxicity, and the substitution of a methyl group for the 2-methoxy group had the same result.

Slotta & Behnisch (1935) found that the combination of dialkylaminoalkyl groups, of the type occurring in synthetic antimalarial compounds of known activity, with hydrocupreine resulted in a decrease in antimalarial activity.

The base 2-amino-5-diethylaminopentane,

$$CH_3—CHNH_2.(CH_2)_3.N(C_2H_5)_2,$$

was found by Fulton (1940) to have a slight action against *P. relictum* in canaries. This compound is interesting on account of its relationship to the side chain of plasmoquine and atebrin, in which compounds one of the hydrogen atoms of the amino group is replaced by 6-methoxyquinoline or 2-methoxy-6-chlor-acridine. I have, however, been unable to confirm its activity upon my strain of *P. relictum*.

17. DRUGS WHICH DIFFER IN THEIR ACTION UPON MALARIA OF MAN, MONKEYS AND BIRDS

Although up to the present no drug of proved worth in the treatment of malaria has been found inactive upon bird malaria, there are a few compounds, of which the chemotherapeutic value is still under discussion, which appear to be more effective upon malaria of man than upon avian malaria.

The successful treatment of malaria with sulphanilamide compounds has been reported by several workers, but the results as a whole are, as Buttle (1939) pointed out, somewhat contradictory. Niven (1938) considered that though sulphanilamide had some action upon *P. falciparum* and *P. vivax* it was so slight that it was of academic interest only. Sinton, Hutton & Shute (1939) found that proseptasine appeared to have a true causal prophylactic action upon the Roumanian strain of *P. falciparum*, but they state that confirmation of the results is needed. As the protective action of a single dose

seems to last less than 48 hr. Sinton (1939) does not consider the discovery to be of much practical value.

Upon some species of monkey malaria, transmitted by blood inoculation, sulphanilamide compounds have a definite curative action. Thus in infections of *P. knowlesi* Das Gupta & Chopra (1938) found that prontosil though it did not sterilize the infection reduced it to a very low grade. Parasites of this species not only disappeared during treatment with M and B 693 (Chopra & Das Gupta, 1939) but they did not reappear after treatment had ended, as they do after atebrin treatment. Sulphanilamide had a curative and strong prophylactic action upon *P. knowlesi* according to Coggeshall (1938), and sulphanilyl sulphanilate some slight therapeutic effect upon acute infections. In mixed infections of *P. knowlesi* and *P. inui* in monkeys sulphanilamide eradicated the virulent *P. knowlesi* and left the host with a chronic infection of the milder *P. inui* (Coggeshall, 1940). In experiments using manometric methods the O_2 consumption of *P. knowlesi* was six times as great as that of *P. inui*; and sulphanilamide, in concentrations less than the curative dose, inhibited the respiration of *P. knowlesi* almost completely, whereas it had no apparent effect upon *P. inui*.

Both sulphanilamide and sulphanilyl sulphanilate were ineffective in the treatment of *P. cathemerium* in canaries, and *P. lophurae* in young chicks (Coggeshall, 1938).

According to Africa, Dy & Soriano (1939), prontosil, given intramuscularly, showed some antimalarial action against *P. relictum* in the Java sparrow (=rice finch). From their tabulated data it appears that treatment was given over a long period, and the results are inconclusive. The Java sparrow is not a good host in which to study the response of *P. relictum* since the wild birds, such as Africa and his collaborators used, are frequently infected with *P. paddae*. Dr Tate and I found an aviary-bred Java sparrow to be resistant to infection with *P. relictum*; no true infection developed after inoculation with heavily parasitized blood, a few parasites being found after long search on two occasions only.

Six successive daily treatments of canaries infected by blood inoculation with *P. relictum*, either with red prontosil given orally or with prontosil soluble given orally in one case and intramuscularly in another, were ineffective in the M.T.D. (My own unpublished results.)

Durand & Villain (1939) found that rubiazol and septolix had no therapeutic action upon heavy infections of *Haemoproteus columbae*.

The antimalarial action of compounds of the sulphanilamide group which have been tested up to the present therefore appears to be strongest against monkey malaria, slighter against malaria of man, and, on the whole, lacking against avian malaria.

In 1938 Glyn-Hughes, Lourie & Yorke (1938) made the interesting discovery that in infections of benign tertian malaria induced in cases of neurosyphilis, the febrile paroxysms ceased and the parasites slowly disappeared

from the blood when treated with 1 : 11-*n*-undecane diamidine dihydrochloride. This compound was found by Christophers & Fulton (1938) to save some of the monkeys infected with *Plasmodium knowlesi* from death in the primary attack, though its action was much slower and less effective than that of atebrin. They found it to be without therapeutic action upon infections of *P. relictum* in canaries, an observation which I can confirm from my own experience. The importance of this compound lies in its chemical structure which differs from that of all known antimalarial compounds. It may therefore lead to profitable research on new lines in the search for antimalarial drugs.

Paludex, a copper hydroxyquinoline sulphonate of soda, which is said to have given favourable results in the treatment of malaria in the Belgian Congo, was found by Rodhain & Hendrix (1937) to be without action upon *P. cathemerium* and *Haemoproteus orizivorae* in birds, and *Plasmodium knowlesi* and *P. gonderi* in monkeys.

Whereas the above compounds were effective in varying degrees upon malaria of man and monkeys but ineffective upon avian malaria, Dimeplasmin, a compound synthesized by the I.G. Farbenindustrie. but of which no description of the constitution was given, was found by Green (1929) to be ineffective, both upon parasites and clinical symptoms, in cases of subtertian, benign tertian, and quartan malaria, though he reports that workers at the Elberfeld laboratories had found it highly effective against avian malaria, its chemotherapeutic index being 1/30.

Two closely related quinoline compounds known as 'R 118' and 'R 123', of whose chemical composition no description has been given, were tested by Collier & Warstadt (1931) and Collier, Warstadt & Krause (1931) upon infections of *P. relictum* in canaries. The chemotherapeutic index of R 118 was 1/250 and that of R 123 even greater; but Sternberg (1934) obtained an index of only 26·6 as compared with 40 for plasmoquine when R 123 was tested against *P. relictum* in *Acanthis linaria* by the same methods. Warstadt & Collier (1931) reported favourably upon its therapeutic action upon infections of benign tertian, quartan and malignant tertian malaria in man, and observed that it was effective against gametocytes as well as schizonts; but no further reports upon the therapeutic value of these compounds appear to have been published.

Hegner, West, Ray & Dobler (1941) have found that hydroxyethylapocupreine, given curatively, has a similar effect upon three species of avian malaria to quinine, but that it is less toxic than that drug. No report upon its action upon malaria of man has yet been published.

18. THE VALUE OF TESTS UPON AVIAN MALARIA IN CHEMOTHERAPY

It is sometimes asked whether, by the use of avian malaria in preliminary tests of antimalarial activity of new synthetic compounds, it is not possible that compounds which might prove effective against malaria of man are dismissed from further investigation because they show no activity against avian

malaria, and whether a method free from this possible error might not be used. Suggestions are sometimes made that monkey malaria should be used instead of avian malaria, because the host is more similar to man physiologically, and therefore the action of drugs is more likely to be similar.

The possibility that a compound may act differently upon avian and human malaria cannot be denied, but its action upon two species of parasite within the same host may also differ, as for example the action of sulphanilamide upon *Plasmodium knowlesi* and *P. inui* in monkeys. In order that no compound which might have an action upon any one of the three species of *Plasmodium* of man might not be overlooked, it would be necessary to test every drug produced by chemists upon each of these three species. If it were possible to infect a suitable animal experimentally with these species, or if continuous cultivation of the parasites was possible, this might be feasible, but the results of cultivation experiments, up to the present, are of no practical value in the selection of antimalarial compounds.

Though Bass & Johns (1912) in one case obtained three generations of parasites, and Thomson & Thomson (1913) four generations, many workers appear to have obtained only the development of the generation of parasites which they put into the medium, and Horovitz & Sautet (1929) regard such results as a 'conservation' rather than a true cultivation. Whereas most workers in this field have used the Bass & Johns method, or modifications of it, in which the infected blood is defibrinated after the addition of dextrose solution and then tubed in small quantities, Hewitt (1938) spread infected blood over the surface of egg slopes and below a layer of serum saline containing over 0·5 % dextrose solution, but he failed to obtain a second generation of parasites or growth after subcultivation. Gavrilov, Bobkoff & Laurencin (1938), using tissue culture methods, obtained only a survival until the 10th day of pigmented parasites of *P. gallinaceum* in non-embryonic bone marrow.

Until a method is devised by which generations of normal parasites can be cultivated indefinitely tests of antimalarial compounds in vitro are not possible; and even if such a method were devised its value as a means of selection of effective compounds would depend upon whether the action of a drug was directly parasiticidal or whether it acted indirectly by the stimulation of a defensive reaction by the host. Even in drugs which were directly parasiticidal the action in vitro might not give a true indication of the action in vivo, as such problems as toxicity to the host, distribution through the host tissues and rapidity of excretion would arise.

Hegner, Shaw & Manwell (1928) and Shaw (1928), postulating that malarial parasites are intracorpuscular, and that any drug which affects them must penetrate the red blood corpuscles, studied the absorption by red blood corpuscles, of chicken or man, of a series of derivatives of quinine of which the therapeutic action upon human and avian malaria was known. Drugs which had a definite antimalarial action upon avian or human malaria were well absorbed by the red blood corpuscles, and those which were not absorbed were

without therapeutic action. Shaw deduced that changes in the structure of quinine which involved the formation of an ionic group which yielded large numbers of ions at *p*H 7·4 brought about a loss in the ability to penetrate the red blood corpuscles. Oxidation of the vinyl group (p. 33) to form quitenine resulted in the complete loss of the ability to penetrate red blood corpuscles, but on esterification the resulting compound was again able to penetrate the corpuscles though to a less extent than the original compound. Quitenine is known to be inactive against avian malaria, whereas in the esters the anti-malarial activity is to some extent regained.

Manometric methods have been used by Christophers & Fulton in studying the respiratory metabolism of *Plasmodium knowlesi*. They (Fulton & Christophers, 1938) found that certain compounds in definite concentrations inhibited the O_2 uptake of the parasites. The effect, to some extent, was parallel to the therapeutic action of the drugs, for those known to be effective against malaria were found to be good inhibitors. Atebrin was 5–10 times more effective as an inhibitor than quinine. By this method the authors were able to estimate the direct toxic action of a compound, but they point out that its action in vivo, even though the mechanism of action may be the same as in vitro, may be complicated by other factors such as distribution in the tissues of the host.

Such experiments, though interesting in the light which they throw upon the mode of action of compounds upon the parasite, do not offer a practical means of testing the antimalarial activity of drugs, or their selection for trial upon malaria of man. Some laboratory animal capable of being infected with a species of *Plasmodium* is necessary for this. There is no guarantee that the similarity in response to any drug would be greater between monkey and human malaria than between avian and human malaria. The cost of monkeys is so great that it prohibits their use in the primary selective tests of new compounds if these are produced in any quantity, as is the case when the search for new drugs is being pursued systematically by chemists; and, in addition to the initial cost of purchase, monkeys are more expensive to feed and to house than birds, and infinitely more difficult to handle.

Although the use of avian malaria is not above criticism as a means of estimating the antimalarial activity of new compounds there is, as yet, no other method which offers as good results, and it has to its credit the discovery of the two compounds, plasmoquine and atebrin, which have proved of great value in the treatment of malaria, and of several others of less certain worth.

It is often asked why, since the response of infections induced by blood inoculation differs widely from that of sporozoite-induced infections, and since the ultimate aim of research upon the chemotherapy of malaria is the discovery of a drug capable of sterilizing naturally acquired, i.e. mosquito-borne infections, infections induced in canaries by blood inoculation are used in the tests upon new drugs. It may be pointed out in reply that, while compounds which have proved to be active against infections induced by blood inoculation should be tested later against sporozoite-induced infections, if plasmoquine

and atebrin had been tested first upon sporozoite-induced infections neither of these compounds would have shown sufficient activity to justify further investigation, yet both are of value in the therapeutics of malaria of man.

19. The definition of some terms as used in this paper

Causal prophylaxis. The destruction, by the action of a drug, of sporozoites after their entry into the vertebrate host but before the commencement of their schizogonic development.

Chemotherapeutic index. The ratio $\frac{\text{Maximum tolerated dose}}{\text{Minimum effective dose}}$.

Chronic infection. An infection which has ceased to be acute. In avian malaria an acute infection passes into a chronic state in which though parasites may be impossible to find by direct microscopic examination of the peripheral blood, their presence can be proved by the infection of clean birds with the blood of the chronic bird.

Clinical prophylaxis. The prevention, by the action of a drug, of the clinical symptoms arising from the presence of malaria parasites in the host.

Curative treatment. The administration of a drug after parasites have appeared in the peripheral blood of the host.

Immunity reaction. A means of testing whether a bird has been sterilized of its infection. Heavily parasitized blood is injected into the bird believed to be sterilized. The development of a normal acute infection indicates that the bird was sterilized, for no acute attack follows superinfection of birds which carry a latent infection.

Incubation period. The period between the injection of parasites into the vertebrate host and their appearance in the red blood corpuscles in the peripheral blood.

Isodiagnosis. A means of testing whether a bird has been sterilized of its infection. The infectivity of blood from a bird believed to have been sterilized of its infection is tested by injection into a clean bird.

Sterilization of infection. The complete eradication of all parasites by the action of a drug.

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THE LARVAL TROMBICULINAE (ACARINA, TROMBIDIIDAE) WITH DESCRIPTIONS OF TWELVE NEW SPECIES

By CHARLES D. RADFORD

(With 106 Figures in the Text)

Introduction

Our knowledge of this interesting group of mites has been built up by the writings of some twenty-two acarologists, of different nationalities, whose papers are published in some four or five languages and have appeared in thirty to forty journals.

It is not possible for every acarologist to have access to this scattered literature, and for some years I have endeavoured to obtain copies of these descriptions. Quite recently I was fortunate in having access to the libraries of the London School of Hygiene and the Zoological Society and have augmented my collection of drawings of the dorsal scuta of these mites.

Having collected a number of specimens of these larval mites during the course of recent years, I wanted the drawings to enable me to determine whether these specimens were new or known species.

Thinking the collection of drawings would be of use to other acarologists I contemplated publication of them in the form of a paper in which all the known species would be listed, with host and locality and, where possible, the dorsal shield would be figured. The new species herein described have additional data.

The group is of medical interest as possible vectors of disease. One species, *Trombicula akamushi* Brumpt, has been proved to be the vector of Japanese River Fever, or tsutsugamushi disease.

Nagayo *et al.* (1921) assert that only one species habitually attacks man, i.e. *Tr. akamushi* Brumpt. 'From the fact that *Tr. akamushi* may infect man by their bite, there is no doubt that this species of mite harbours the virus of the tsutsugamushi disease in itself and transmits it when it has occasion to feed. It has been proven by animal experiments with *pallida* and *intermedia* that they may also carry the virus in themselves. But as they do not bite man it may be said that there is no direct relationship between them and the occurrence of human tsutsugamushi disease' (p. 584).

Ewing (1937) considers that the five species listed by Nagayo *et al.* (1921), *Tr. akamushi*, *pallida*, *palpalis*, *intermedia* and *scutellaris*, are only seasonal varieties of one species (*akamushi*).

Nagayo *et al.* (1921, p. 570) state: 'Our researches on this problem in successive years threw much light upon the morphology as well as the biology of these

mites and we are now able to state positively that there are up to the present at least five species of tsutsugamushi which differ not only morphologically, but also biologically, from each other and show different seasonal prevalence.' (P. 571): 'Up to the present we have hatched the larvae of *Tr. akamushi*, *pallida* and *palpalis* from their adults artificially in bottles.' (P. 577): 'In the breeding experiments we have obtained nymphs of four species: *Tr. akamushi*, *pallida*, *palpalis* and *scutellaris*'.... 'So far we have succeeded in our breeding experiments in obtaining adults of *Tr. akamushi*, *pallida* and *palpalis*.'

On 538 voles examined, according to the figures given by Nagayo, there were

4131 *Tr. akamushi*	3863 *Tr. palpalis*
14470 *Tr. pallida*	1849 *Tr. intermedia*

The amount of work involved in the compilation of this paper by Nagayo *et al.* leaves no room for doubt that the specific characters are sufficiently distinct to warrant specific rank to these five species.

Walch (1923) points to *Tr. deliensis* as the vector of the disease in Sumatra.

Fonseca (1932*b*, 1935) described and figured two new species (*Tr. travassosi* and *Tr. ewingi*) in which the antero-lateral setae were represented by tooth-like projections. In one species *Tr. ewingi* Fonseca the median seta is normal, whilst in *Tr. travassosi* Fonseca the median seta is also similarly a tooth-like projection.

In view of this unusual feature, antero-lateral setae replaced by tooth-like projections only at present recorded in these two Brazilian species, I think it advisable to raise a new genus for which the name *Fonsecia* is proposed with *F. ewingi* as the type species.

The key to the genera, published by Ewing (1937) is here inserted as an appendix.

The drawings have been copied from various papers (original papers where possible), and only the new species are from my own slides.

To Prof. P. A. Buxton, London School of Hygiene, I am indebted for the Library facilities accorded to me at that Institute. Also for arranging similar facilities for me at the Zoological Society. To the Library Staff at both these Institutions I wish to express my thanks for their kind assistance.

Dr Flavio da Fonseca, S. Paulo, Brazil, has given much help by the exchange of his interesting papers on Acari, also Dr A. C. Oudemans, Arnhem, Netherlands, who, in addition, has given helpful advice in the initial stages of this paper.

Col. A. E. Hamerton, Pathologist, London Zoo, has for some years sent me a number of interesting specimens, and in one tube containing the lungs or air sac of a Corn snake (*Elaphe guttata*) there was a specimen of a larval trombidiid, herein described as *Tr. hamertoni* n.sp.

The South African Zoological Survey Committee, Pretoria, has, through Dr P. J. du Toit, contributed a number of interesting specimens. Dr D. H. S. Davis, Pretoria, sent from Sierra Leone some specimens of interest. Dr H. H.

Parker, Spotted Fever Laboratory, Hamilton, Montana, has contributed a number of new specimens.

The host nomenclature has been revised by the British Museum (Zool. Dept.), and I wish to thank M. A. C. Hinton, Esq., and his assistants for their help.

To all these persons I wish to acknowledge thanks for their specimens or helpful advice.

Type slides of the new species are deposited in the British Museum (Nat. Hist.).

FAMILY TROMBIDIIDAE Leach, 1814

Genus **Trombicula** Berlese, 1905

Trombicula batatus L., 1758.
Host. ?.
Locality. Surinam.

Trombicula bisignata Ewing, 1929.
Host. Meadow mouse (*Microtus pennsylvanicus pennsylvanicus* Ord.).
Locality. Mount Katahdin, Maine.

Trombicula irritans Riley, 1873.
Host. Man.
Locality. United States.

Trombicula guineense Bruyant & Joyeux, 1913 (Fig. 1).
Host. Domestic fowl and two monkeys (Patas monkey, *Erythrocebus patas pyrrhonotus* Hembr. & Ehr., and Green monkey, *Cercopithecus aethiops sabaeus* Linn.).
Locality. French Guiana.

Trombicula japa Ribeyro & Bambaren, 1932.
Host. ?.
Locality. Peru.

Trombicula microti Ewing, 1928.
Host. Big-footed meadow mouse (*Microtus richardsoni macropus* Merriam).
Locality. Wyoming.

Trombicula oregonensis Ewing, 1929.
Host. American mole (*Scapanus* sp.).
Locality. Corvallis, Oregon.

Trombicula shannoni Ewing, 1929.
Host. House cat (*Felis domesticus*).
Locality. Verrugas Cañon, Lima, Peru.

Trombicula thomasi Oudemans, 1910 (Fig. 2).
Host. Mouse (*Chraeomys jelskii* Thomas).
Locality. Central Peru.

Trombicula whartoni Ewing, 1929.
Host. A bird.
Locality. Summerville, South Carolina.

Trombicula blarinae Ewing, 1931 (Fig. 3).
Host. Short-tailed shrew (*Blarina brevicauda* Say.).
Locality. Washington, D.C.

Trombicula piercei Ewing, 1933.
Host. Horseshoe bat (*Hipposideros diadema griseus* Meyen).
Locality. Barrio Buyog, Philippine Islands.

Trombicula hirsti Sambon, 1927 (Fig. 4).
Host. Man.
Locality. Townsville, North Queensland.

Trombicula wichmanni Oudemans, 1905.
Host. Crowned pigeon (*Goura cristata* Vroeg), and man.
Locality. New Guinea, N. Celebes.

Trombicula akamushi Brumpt, 1910 (Fig. 5).
Host.
Locality.

Trombicula pseudo akamushi Hatori, 1919.
Host.
Locality.

Trombicula deliensis Walch, 1923 (Fig. 6).
Host. Man and rats.
Locality. Deli, Sumatra.

Trombicula pallida Nagayo, 1919 (Fig. 7).
Host. Vole.
Locality. Yachi, Yamagata Prefecture, Japan.

Trombicula palpalis Nagayo, 1919 (Fig. 8).
Host. Vole.
Locality. Yachi, Yamagata Prefecture, Japan.

Trombicula scutellaris Nagayo, 1920 (Fig. 9).
Host. Vole.
Locality. Yachi, Yamagata, Prefecture, Japan.

Trombicula intermedia Nagayo, 1920 (Fig. 10).
Host. Vole.
Locality. Arato, Yamagata Prefecture, Japan.

Trombicula brumpti Lahille, 1927.
Host.
Locality.

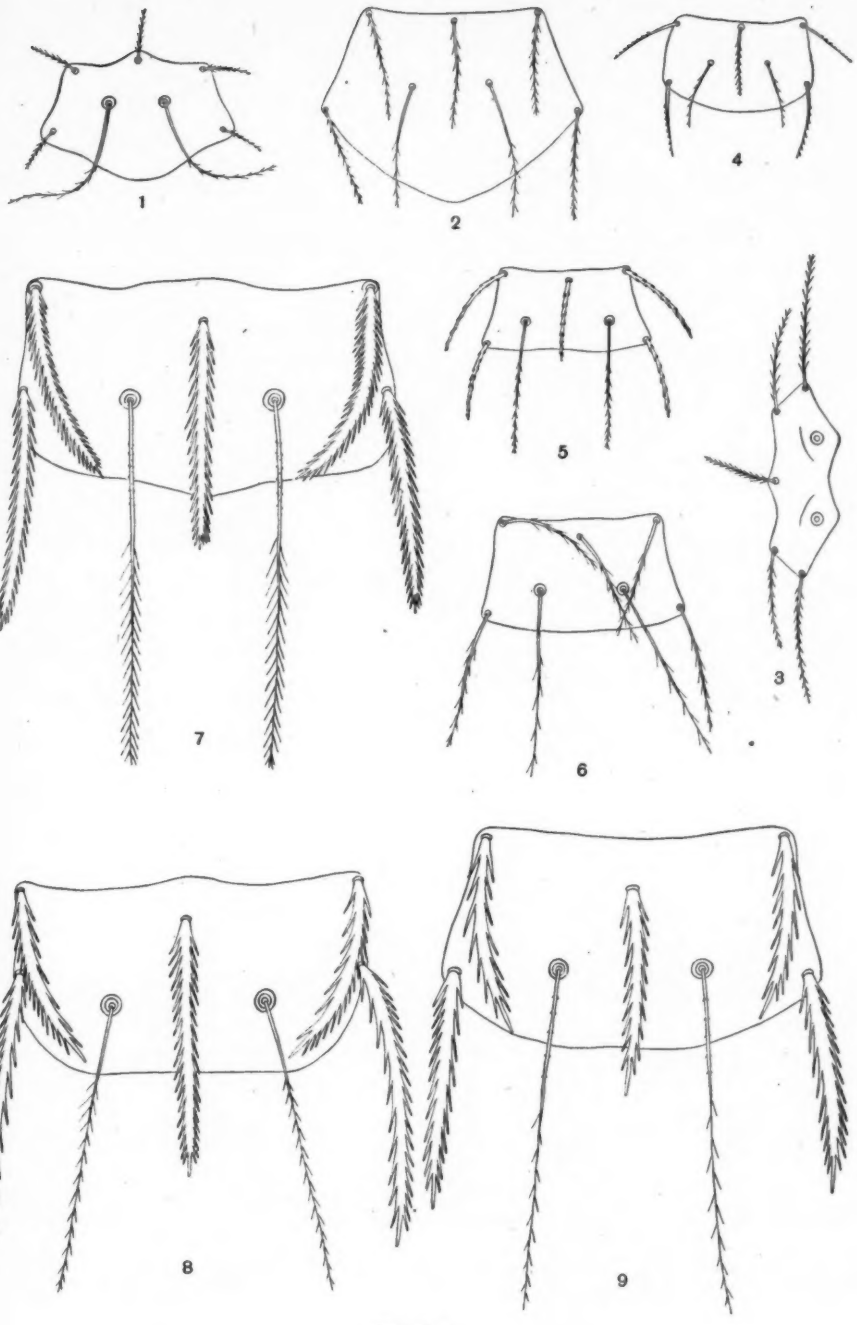
Trombicula gliricolens Hirst, 1915 (Fig. 11).
Host. Black rat (*Rattus rattus* L.).
Locality. Calcutta, India.

Trombicula acuscutellaris Walch, 1923 (Fig. 12).
Host. Rat.
Locality. Deli, Sumatra.

Trombicula densipiliata Walch, 1923 (Fig. 13).
Host. ?.
Locality. Deli, Sumatra.

Legends to figs. 1–9

1. *Trombicula guineense* Bruyant & Joyeux. 2. *Trombicula thomasi* Oudemans. 3. *Trombicula blarinae* Ewing. 4. *Trombicula hirsti* Sambon. 5. *Trombicula akamushi* Brumpt. 6. *Trombicula deliensis* Walch. 7. *Trombicula pallida* Nagayo. 8. *Trombicula palpalis* Nagayo. 9. *Trombicula scutellaris* Nagayo.



Figs. 1–9

Trombicula isshikii Sugimoto, 1938 (Fig. 14).
Host. Latham's snipe (*Capella hardwickii* Gray).
Locality. Naikoshō, Shichiseigun, Prefecture, Taihoku, Formosa.

Trombicula ardeae Tragardh, 1904 (Fig. 15).
Host. Heron (*Ardea cinerea* L.).
Locality. White Nile.

Trombicula fahrenholzi Oudemans, 1910 (Fig. 16).
Host. Long-fingered bat (*Miniopterus schreibersi* Kuhl.).
Locality. Bremen.

Trombicula tragardhi Oudemans, 1910 (Fig. 17).
Host. Grivet monkey (*Cercopithecus aethiops aethiops* Linn.).
Locality. White Nile.

Trombicula muris Oudemans, 1910.
Host. Wood mouse (*Apodemus sylvaticus* L.).
Locality. Bremen.

Trombicula minutissimum Oudemans, 1910 (Fig. 18).
Host. Bat.
Locality. Durban, South Africa.

Trombicula sulae Oudemans, 1910 (Fig. 19).
Host. Gannet (*Morus capensis* Lichtenstein).
Locality. West Africa.

Trombicula setosa Ewing, 1937.
Host. Cotton mouse (*Peromyscus gossypinus gossypinus* Le Conte).
Locality. Okefinokee Swamp, Georgia.

Trombicula cynos Ewing, 1937.
Host. Raccoon (*Procyon lotor* Linn.).
Locality. Ithaca, New York.

Trombicula dasyproctae Ewing, 1937.
Host. Aguti (*Dasyprocta punctata* Gray).
Locality. Capira, Panama.

Trombicula mexicana Ewing, 1937.
Host. Bat.
Locality. San Luis Potosi, Mexico.

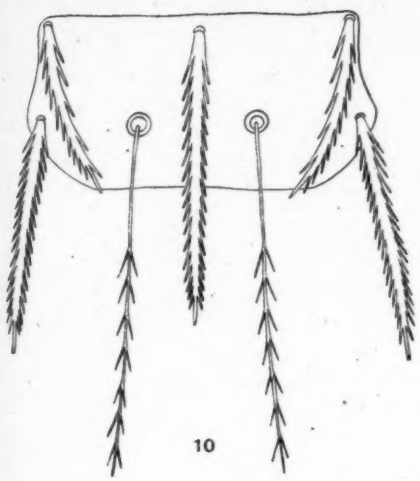
Trombicula nigeriensis Ewing, 1928.
Host. Nigerian squirrel (*Funisciurus auriculatus oliviae* Dollman).
Locality. Nigeria, Africa.

Trombicula centropodia Ewing, 1928.
Host. Cuckoo (*Centropus* sp.).
Locality. Gbanga, Liberia, Africa.

Trombicula vanommereni Schierbeek, 1937.
Host. Man and lizards.
Locality. Paramaribo, Dutch Guiana.

Legends to figs. 10–18

10. *Trombicula intermedia* Nagayo. 11. *Trombicula gliricolens* Hirst. 12. *Trombicula acuscutellaris* Walch. 13. *Trombicula densipiliata* Walch. 14. *Trombicula isshikii* Sugimoto. 15. *Trombicula ardeae* Tragardh. 16. *Trombicula fahrenholzi* Oudemans. 17. *Trombicula tragardhi* Oudemans. 18. *Trombicula minutissimum* Oudemans.



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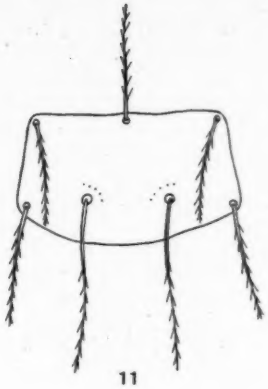

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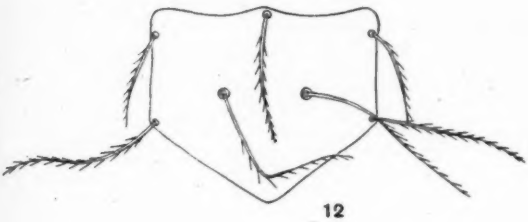

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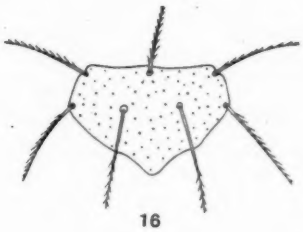

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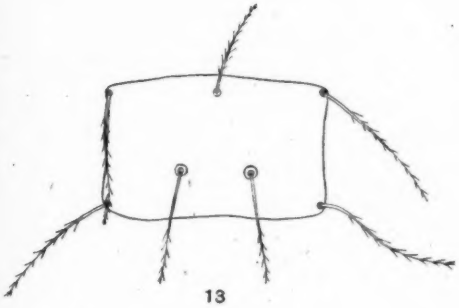

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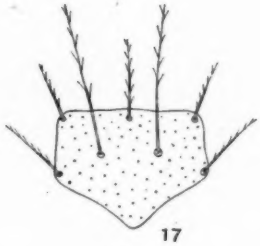

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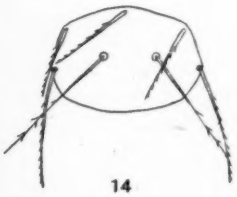

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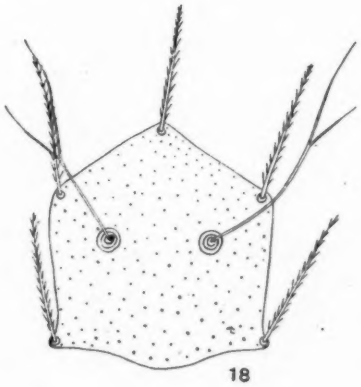


18

Figs. 10–18

Trombicula novae-hollandiae Hirst, 1929 (Fig. 20).
Host. Grey's rat (*Rattus greyi* Gray).
Locality. Kangaroo Island, South Australia.

Trombicula buloloensis Gunther, 1939 (Fig. 21).
Host. Man, Cockerell's bandicoot (*Echymipera cockerelli* Ramsay), bush fowl (*Megapodius duperreyi*), bush turkeys (*Talegallus jobiensis*), cassawaries (*Casaurius casaurius* L.).
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Trombicula rioi Gunther, 1939 (Fig. 22).
Host. Bush fowl (*Megapodius duperreyi*).
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Trombicula spicea Gater, 1932 (Fig. 23).
Host. Malay grey rat (*Rattus canus malaisia* Kloss).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Trombicula munda Gater, 1932 (Fig. 24).
Host. Malaysian house rat (*Rattus rattus diardi* Jentink).
Locality. Kuala Lumpur, Selangor, Federated Malay States.

Trombicula hastata Gater, 1932 (Fig. 25).
Host. Spiny-backed rat (*Rattus surifer surifer* Miller).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Trombicula cervulicola Ewing, 1931 (Fig. 26).
Host. Barking deer or muntjak (*Muntiacus muntjak aureus* H. Smith).
Locality. Muktesar, Kumaun, India.

Trombicula autumnalis Shaw, 1790 (Fig. 27).
Host.
Locality.

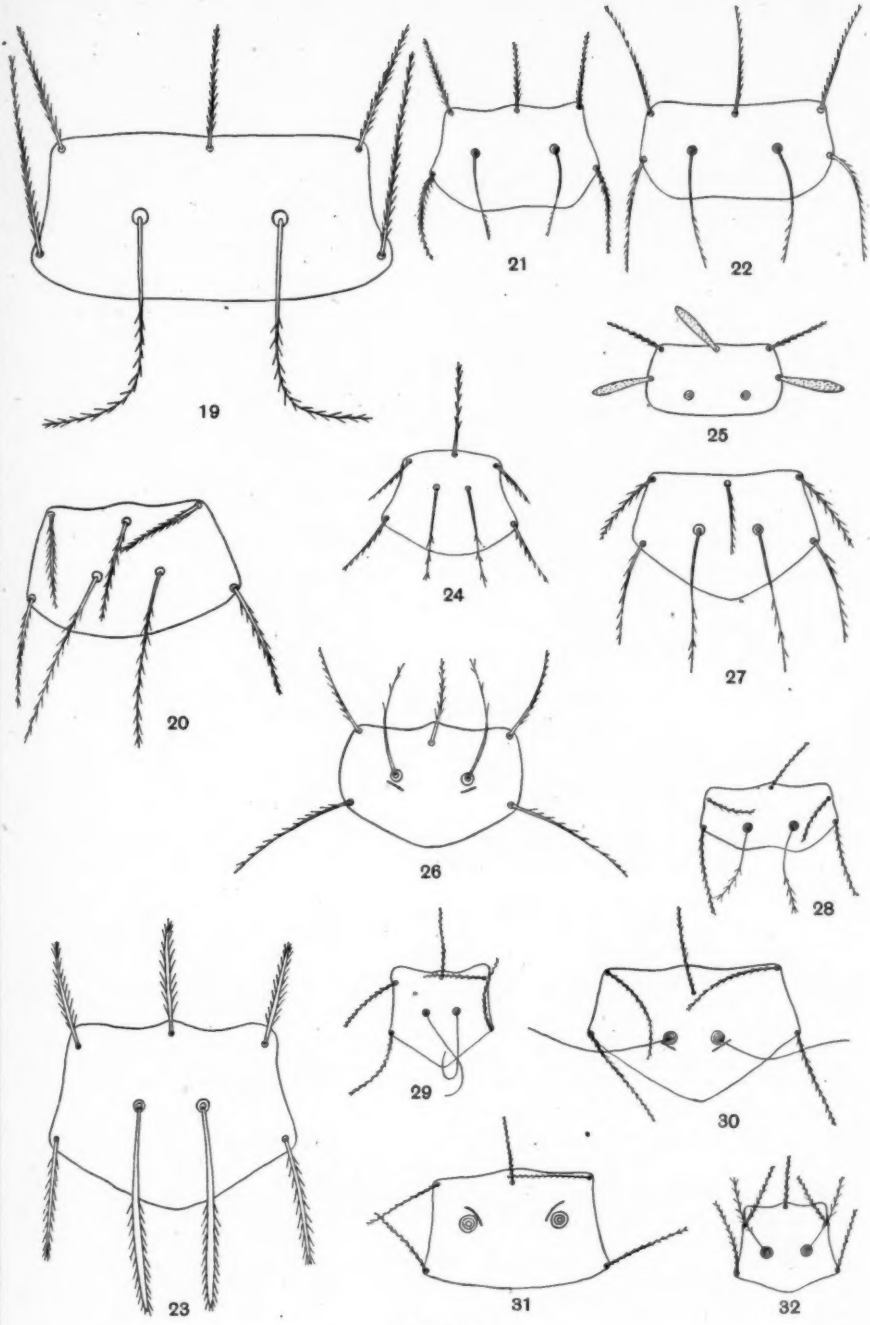
Trombicula hamertoni n.sp. (Fig. 28).
Host. Corn snake ? (*Elaphe guttata*).
Locality. London Zoo, 8 March 1939.
Dorsal setae about 22, arranged in rows of 6, 6, 6, 4.
Measurements: 0·37 × 0·33 mm.

Trombicula cynictia n.sp. (Fig. 29).
Host. Meerkat (*Cynictis penicillata ogilbyi* A. Smith).
Locality. Hoopstad, Orange Free State, South Africa, 30 June 1938.
Dorsal setae about 31, arranged in rows of 8, 8, 7, 4, 4.
Measurements: 0·39 × 0·25 mm.

Trombicula parkeri n.sp. (Fig. 30).
Host. Cony.
Locality. Antonito, Colorado, 9 September 1931.
Dorsal setae about 40, in rows of 10, 10, 10, 10.
Measurements: 0·45 × 0·35 mm.

Legends to figs. 19–32

19. *Trombicula sulae* Oudemans. 20. *Trombicula novae-hollandiae* Hirst. 21. *Trombicula buloloensis* Gunther. 22. *Trombicula rioi* Gunther. 23. *Trombicula spicea* Gater. 24. *Trombicula munda* Gater. 25. *Trombicula hastata* Gater. 26. *Trombicula cervulicola* Ewing. 27. *Trombicula autumnalis* Shaw. 28. *Trombicula hamertoni* n.sp. 29. *Trombicula cynictia* n.sp. 30. *Trombicula parkeri* n.sp. 31. *Trombicula praomyia* n.sp. 32. *Trombicula mastomyia* n.sp.



Figs. 19–32

Trombicula praomyia n.sp. (Fig. 31).
Host. Short-haired rat (*Praomys tullbergi* Thomas).
Locality. Sierra Leone, 26 February 1937.
Dorsal setae about 66, arranged in irregular rows.
Measurements: 0·55 × 0·35 mm.

Trombicula mastomyia n.sp. (Fig. 32).
Host. Multimammate rat (*Mastomys coucha erythroleucus* Temm.).
Locality. Freetown, Sierra Leone, 27 January 1936.
Dorsal setae about 38, arranged in rows of 6, 8, 6, 8, 6, 4.
Measurements: 0·46 × 0·28 mm.

Genus **Gahrliepia** Oudemans, 1912

Gahrliepia nanus Oudemans, 1910 (Fig. 33).
Host. Bat.
Locality. Durban, South Africa.

Gahrliepia rustica Gater, 1932 (Fig. 34).
Host. Spiny-backed rat (*Rattus surifer surifer* Miller).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Gahrliepia cetrata Gater, 1932 (Fig. 35).
Host. Malay giant rat (*Rattus edwardsi ciliatus* Bonhote).
Locality. Fraser's Hill, Pahang, Federated Malay States.

Gahrliepia turmalis Gater, 1932.
Host. Large spiny-backed rat (*Rattus sabanus vociferans* Miller).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Genus **Walchia** Ewing, 1931

Walchia lewthwaitei Gater, 1932 (Fig. 36).
Host. Rat (*Rattus rattus* [*diardi* ?]).
Locality. Kuala Lumpur, Selangor, Federated Malay States.

Walchia enode Gater, 1932.
Host. Müller's rat (*Rattus mülleri validus* Miller).
Locality. Sungei Buloh, Selangor, Federated Malay States.

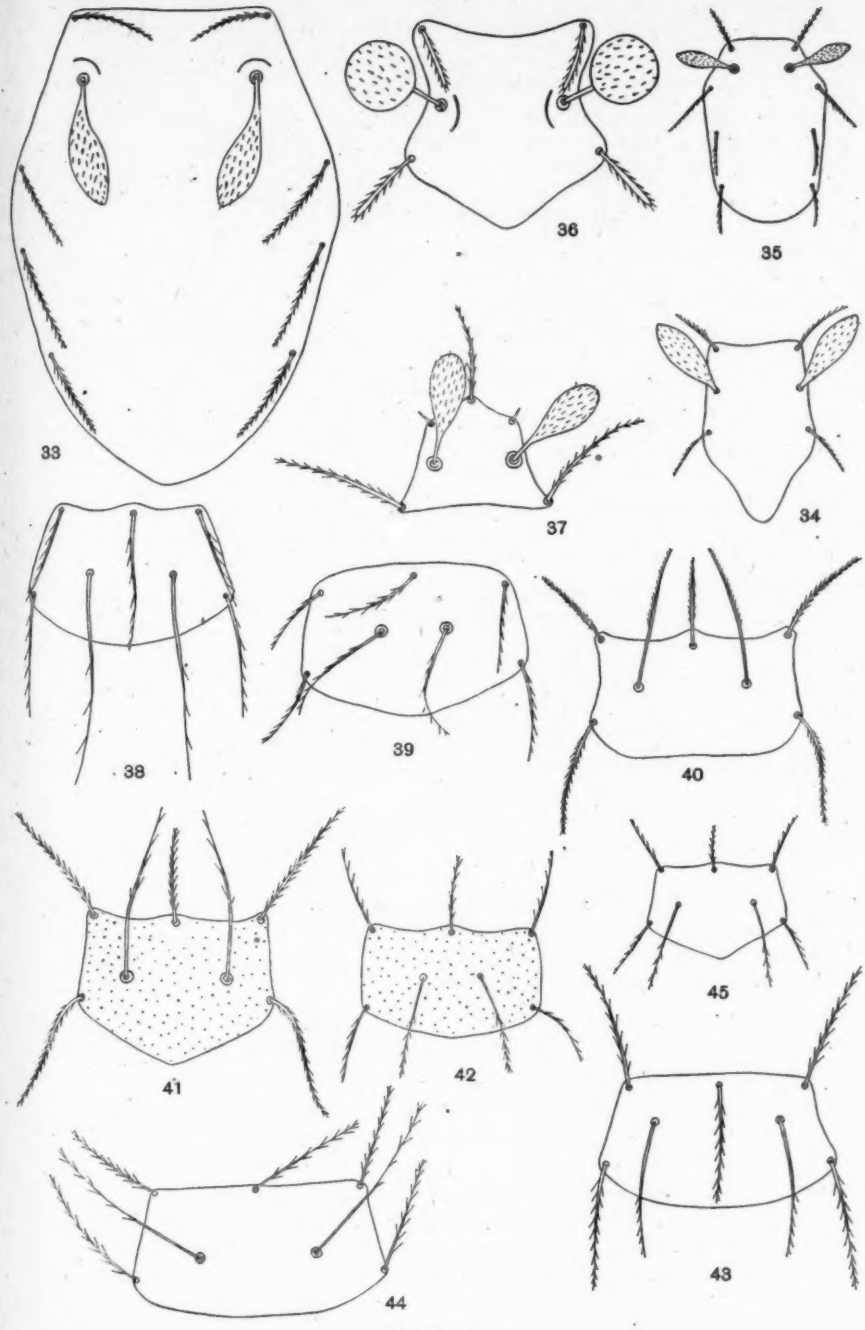
Walchia pingue Gater, 1932.
Host. Malay giant rat (*Rattus edwardsi ciliatus* Bonhote).
Locality. Fraser's Hill, Pahang, Federated Malay States.

Walchia glabrum Walch, 1927.
Host.
Locality.

Walchia morobensis Gunther, 1939.
Host. Rats (*Rattus browni* Alston and *R. ringens* Peters & Doria).
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Legends to figs. 33–45

33. *Gahrliepia nanus* Oudemans. 34. *Gahrliepia rustica* Gater. 35. *Gahrliepia cetrata* Gater. 36. *Walchia lewthwaitei* Gater. 37. *Doloisia synoti* Oudemans. 38. *Eutrombicula hominis* Ewing. 39. *Eutrombicula flui* Van Theil. 40. *Eutrombicula butantanensis* Fonseca. 41. *Eutrombicula ophidica* Fonseca. 42. *Eutrombicula bruyanti* Oudemans. 43. *Eutrombicula goldii* Oudemans. 44. *Eutrombicula helleri* Oudemans. 45. *Eutrombicula tinami* Oudemans.



Figs. 33–45

Genus **Doloisia** Oudemans, 1910

Doloisia synoti Oudemans, 1910 (Fig. 37).
Host. Barbastelle bat (*Barbastella barbastellus* Schreber).
Locality. Bremen, Germany.

Genus **Eutrombicula** Ewing, 1938

Eutrombicula alfreddugesi Oudemans, 1910.
Host. Man.
Locality. Mexico.

Eutrombicula hominis Ewing, 1933 (Fig. 38).
Host. Man.
Locality. Aguabuenas, Rep. Panama.

Eutrombicula flui Van Theil, 1930 (Fig. 39).
Host. Man.
Locality. Surinam.

Eutrombicula butantanensis Fonseca, 1932 (Fig. 40).
Host. Man.
Locality. Butantan, Sao Paulo, Brazil.

Eutrombicula ophidica Fonseca, 1932 (Fig. 41).
Host. Merrem's Boipeva snake (*Ophis merremii* Wagler).
Locality. Sao Paulo, Brazil.

Eutrombicula tropica Ewing, 1925.
Host. Lizard (*Anaidia bitaeniata* Blgr.).
Locality. Chama River, Venezuela.

Eutrombicula insularis Ewing, 1925.
Host. Lizard (*Anolis cybotes* Cope).
Locality. Santo Domingo.

Eutrombicula brasiliensis Ewing, 1925.
Host. ??.
Locality. Manáos, Brazil.

Eutrombicula myotis Ewing, 1929.
Host. Little brown bat (*Myotis lucifugus lucifugus* Le Conte).
Locality. Mount Katahdin, Maine.

Eutrombicula panamensis Ewing, 1925.
Host. Chiriqui cotton rat (*Sigmodon hispidus chiriquensis* Allen).
Locality. Panama.

Eutrombicula bruyanti Oudemans, 1910 (Fig. 42).
Host. Naked-tailed opossum (*Metachirus opossum* Seba.).
Locality. South Brazil.

Eutrombicula goldii Oudemans, 1910 (Fig. 43).
Host. Aguti (*Dasyprocta aguti* L.).
Locality. Brazil.

Eutrombicula helleri Oudemans, 1911 (Fig. 44).
Host. A beetle (*Passalus* sp.).
Locality. Surinam.

Eutrombicula tinami Oudemans, 1910 (Fig. 45).
Host. Banded tinamou (*Crypturus noctivagus* Wied.).
Locality. Brazil.

Eutrombicula harperi Ewing, 1931.
Host. Woodland jumping mouse (*Napaeozapus insignis* Miller).
Locality. Heart Lake, Essex County, N.Y.

Eutrombicula dunni Ewing, 1931 (Fig. 46).
Host. Aguti (*Dasyprocta punctata nuchalis* Goldman).
Locality. Chiriqui, Panama.

Eutrombicula gurneyi Ewing, 1937.
Host. Blue-tailed skink (*Eumeces fasciatus* L.).
Locality. Priest Bridge, Patuxent River, Maryland.

Eutrombicula cavicola Ewing, 1931.
Host. ??.
Locality. Reynolds Cave, Kentucky.

Eutrombicula yorkei Sambon, 1928 (Fig. 47).
Host. Daudin's Hyla (*Hyla rubra* Daud.).
Locality. Urucum, Matto Grosso, Brazil.

Genus **Gateria** Ewing, 1938

Gateria fletcheri Gater, 1932 (Fig. 48).
Host. Malaysian house rat (*Rattus rattus diardi* Jentink).
Locality. Kuala Lumpur, Selangor, Federated Malay States.

Gateria ciliata Gater, 1932 (Fig. 49).
Host. Müller's rat (*Rattus mülleri validus* Miller).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Gateria rutila Gater, 1932 (Fig. 50).
Host. Müller's rat (*Rattus mülleri validus* Miller).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Genus **Schöngastia** Oudemans, 1910

Schöngastia vandersandei Oudemans, 1905.
Host. Man.
Locality. New Guinea.

Schöngastia pillersi Sambon, 1928 (Fig. 51).
Host. Frog (*Phrynobatrachus natalensis* Smith).
Locality. Upper reaches of White Volta Valley, Ashanti, West Africa.

Schöngastia cercopitheci Tragardh, 1904 (Fig. 52).
Host. Grivet monkey (*Cercopithecus aethiops aethiops* Linn.).
Locality. White Nile.

Schöngastia trouessarti Oudemans, 1910 (Fig. 53).
Host. Naked-tailed opossum (*Metachirus opossum* Seba.).
Locality. Brazil.

Schöngastia antipodianum Hirst, 1929 (Fig. 54).
Host. Grey's rat (*Rattus greyi* Gray).
Locality. Kangaroo Island, South Australia.

Schöngastia coorongense Hirst, 1929 (Fig. 55).
Host. Ears of a rodent.
Locality. Robe, South Australia.

Schöngastia madagascariensis Sambon, 1928 (Fig. 56).
Host. Frog (*Mantidactylus luteus* Methuen & Hewitt).
Locality. Madagascar.

Schöngastia dasycerci Hirst, 1929 (Fig. 57).
Host. Crested tailed Phascogale *Dasycercus cristicaudata*.
Locality. Ooldea, South Australia.

Schöngastia westraliense Womersley, 1934 (Fig. 58).
Host. Cat.
Locality. Greenbushes, West Australia.

Schöngastia petrogale Womersley, 1934 (Fig. 59).
Host. Wallaby.
Locality. Musgrave Ranges, South Australia.

Schöngastia aethiopica Hirst, 1926 (Fig. 60).
Host. Small grey bat.
Locality. Accra.

Schöngastia blestowei Gunther, 1939 (Fig. 61).
Host. Bush fowl (*Megapodius duperreyi*).
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Schöngastia jamesi Gunther, 1939 (Fig. 62).
Host. Bush fowl (*M. duperreyi*), Cockerell's bandicoot (*Echymipera cockerelli* Ramsay).
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Schöngastia vieta Gater, 1932 (Fig. 63).
Host. Malaysian house rat (*Rattus rattus diardi* Jentink).
Locality. Kuala Lumpur, Selangor, Federated Malay States.

Genus **Schöngastiella** Hirst, 1915

Schöngastiella bengalensis Hirst, 1915 (Fig. 64).
Host. Black rat (*Rattus rattus* L.).
Locality. Calcutta, India.

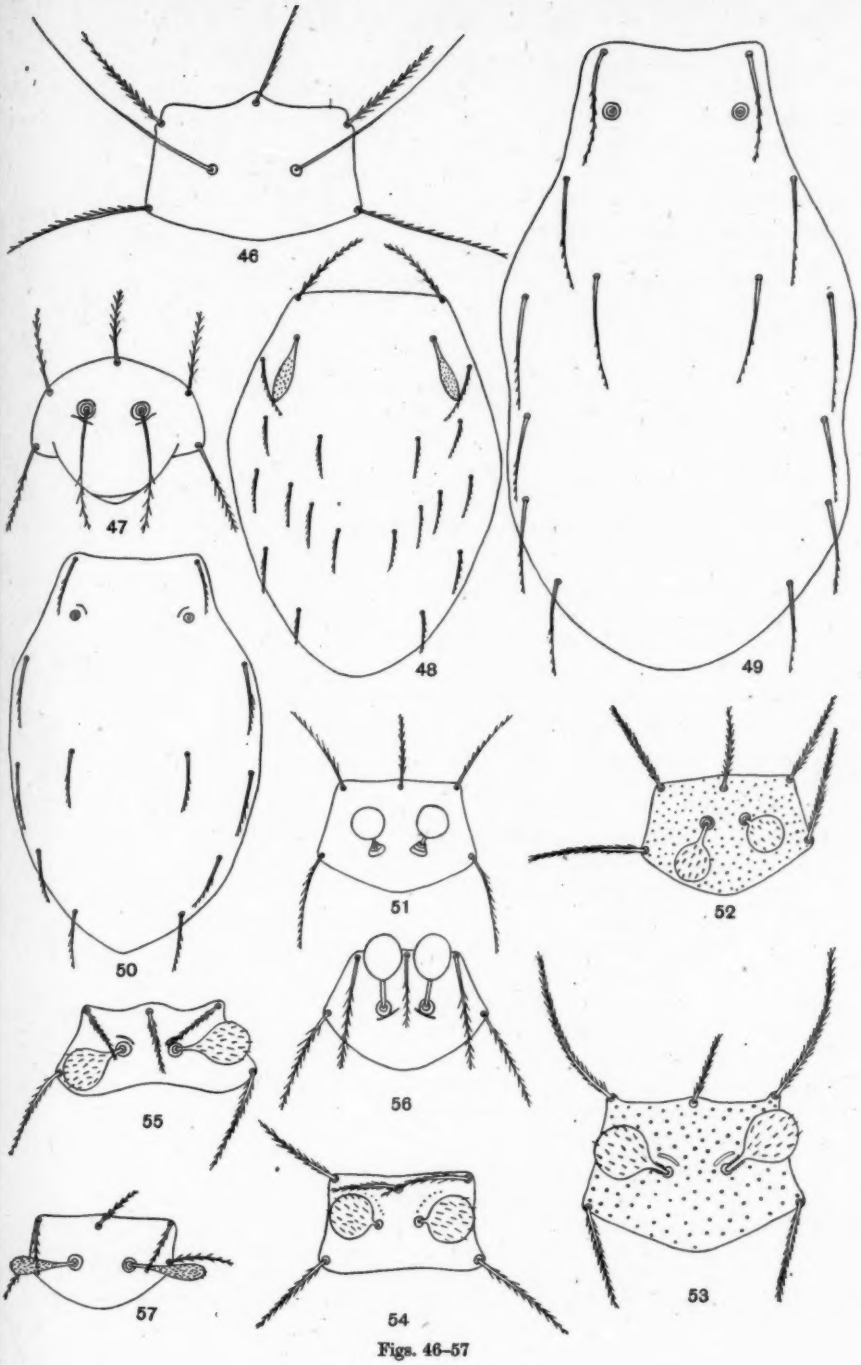
Genus **Leeuwenhoekia** Oudemans, 1911

Leeuwenhoekia verduni Oudemans, 1910 (Fig. 65).
Host. Naked-tailed opossum (*Metachirus opossum* Seba.).
Locality. Brazil.

Leeuwenhoekia polydiscum Oudemans, 1910 (Fig. 66).
Host. Bat.
Locality. Durban.

Legends to figs. 46–57

46. *Eutrombicula dunni* Ewing. 47. *Eutrombicula yorkei* Sambon. 48. *Gateria fletcheri* Gater. 49. *Gateria ciliata* Gater. 50. *Gateria rutila* Gater. 51. *Schöngastia pillersi* Sambon. 52. *Schöngastia cercopitheci* Tragardh. 53. *Schöngastia trouessarti* Oudemans. 54. *Schöngastia antipodianum* Hirst. 55. *Schöngastia coorongense* Hirst. 56. *Schöngastia madagascariensis* Sambon. 57. *Schöngastia dasycerci* Hirst.



Figs. 46–57

Leeuwenhoekia jaegerskioeldi Oudemans, 1911 (Fig. 67).
Host. Under stones.
Locality. Cairo.

Leeuwenhoekia australiensis Hirst, 1925 (Fig. 68).
Host. Man.
Locality. Sydney, New South Wales, Australia.

Leeuwenhoekia gateri n.sp. (Fig. 69).
Host. Gerbille (*Tatera brantsii* A. Smith).
Locality. Holfontein, Orange Free State, South Africa, 18 October 1938.
Dorsal setae about 156–160.
Measurements: 0·33 × 0·31 mm.

Genus **Hannemania** Oudemans, 1911

Hannemania argentina Lahille, 1927.
Host.
Locality.

Hannemania dunni Sambon, 1928 (Fig. 70).
Host. Salamander (*Desmognathus fuscus fuscus* Rafinesque).
Locality. U.S.A.

Hannemania edwardsi Sambon, 1928 (Fig. 71).
Host. Toad (*Bufo variegatus* Gunther).
Locality. Puerto Blest, Argentine Andes.

Hannemania eltoni Sambon, 1928 (Fig. 72).
Host. Southern leopard frog (*Rana sphenocephala* Cope).
Locality. San Antonio, Texas.

Hannemania hobdayi Sambon, 1928.
Host. Frog (*Pleurodema bufonina* Bell).
Locality. Rio Negro, Argentine.

Hannemania newsteadi Sambon, 1928 (Fig. 73).
Host. Daudin's hyla (*Hyla rubra* Daud.).
Locality. Urucum, Matto Grosso, Brazil.

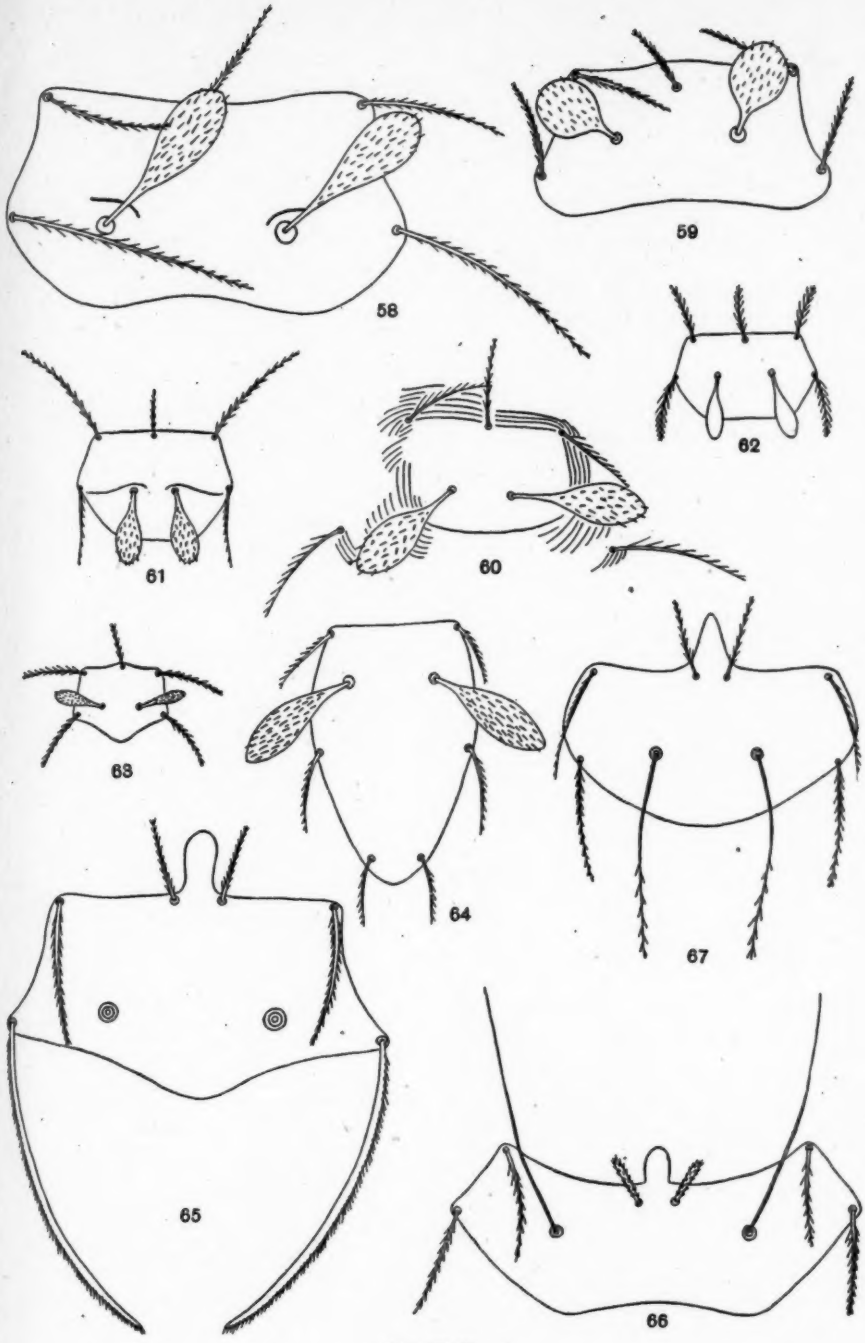
Hannemania pattoni Sambon, 1928.
Host. Frog (*Eupsophus taeniatus* Girard).
Locality. Temuco, Chile.

Hannemania stephensi Sambon, 1928 (Fig. 74).
Host. Frog (*Eleutherodactylus gollmeri* Peters).
Locality. Tombador, Matto Grosso, Brazil.

Hannemania samboni Ewing, for *H. argentina* Sambon, name preoccupied.
Host. Bibron's frog (*Pleurodema bibroni* Tschudi).
Locality. Rio Negro, Argentine.

Legends to figs. 58–67

58. *Schöngastia westraliense* Womersley. 59. *Schöngastia petrogale* Womersley. 60. *Schöngastia aethiopica* Hirst. 61. *Schöngastia blestowei* Gunther. 62. *Schöngastia jamesi* Gunther. 63. *Schöngastia vieta* Gater. 64. *Schöngastiella bengalensis* Hirst. 65. *Leeuwenhoekia verduni* Oudemans. 66. *Leeuwenhoekia polydiscum* Oudemans. 67. *Leeuwenhoekia jaegerskioeldi* Oudemans.



Figs. 58–67

Hannemania hylae Ewing, 1925.
Host. Tree frog (*Hyla arenicolor* Cope).
Locality. Cottonwood Creek, San Diego, California.

Hannemania hylodeus Oudemans, 1910 (Fig. 75).
Host. Frog (*Hylodes* sp.).
Locality. Brazil.

Hannemania hirsuta Ewing, 1931 (Fig. 76).
Host. Californian pocket-mouse (*Perognathus californicus californicus* Merriam).
Locality. Berkeley, California.

Hannemania penetrans Ewing, 1931.
Host. Leopard frog (*Rana pipiens* Schreiber).
Locality. Great Falls of Potomac, Virginia.

Hannemania hepatica Fonseca, 1935 (Fig. 77).
Host. Whistling frog (*Leptodactylus ocellatus* L.).
Locality. Butantan, Sao Paulo, Brazil.

Hannemania ochotrona n.sp. (Fig. 78).
Host. Pika (*Ochotrona* sp.).
Locality. Rassis Hole, Montana, 2 December 1933.
Dorsal setae about 168–174.
Measurements: 0·37 × 0·25 mm.

Genus **Neoschöngastia** Ewing, 1929

Neoschöngastia americana Hirst, 1921 (Fig. 79).
Host. Domestic fowl (*Gallus domesticus*).
Locality. Dallas, Texas.

Neoschöngastia californica Ewing, 1925.
Host. Ground squirrel.
Locality. Topaz, California.

Neoschöngastia peromysci Ewing, 1929.
Host. Northern white-footed mouse (*Peromyscus leucopus noveboracensis* Fischer).
Locality. Sturbridge, Massachusetts.

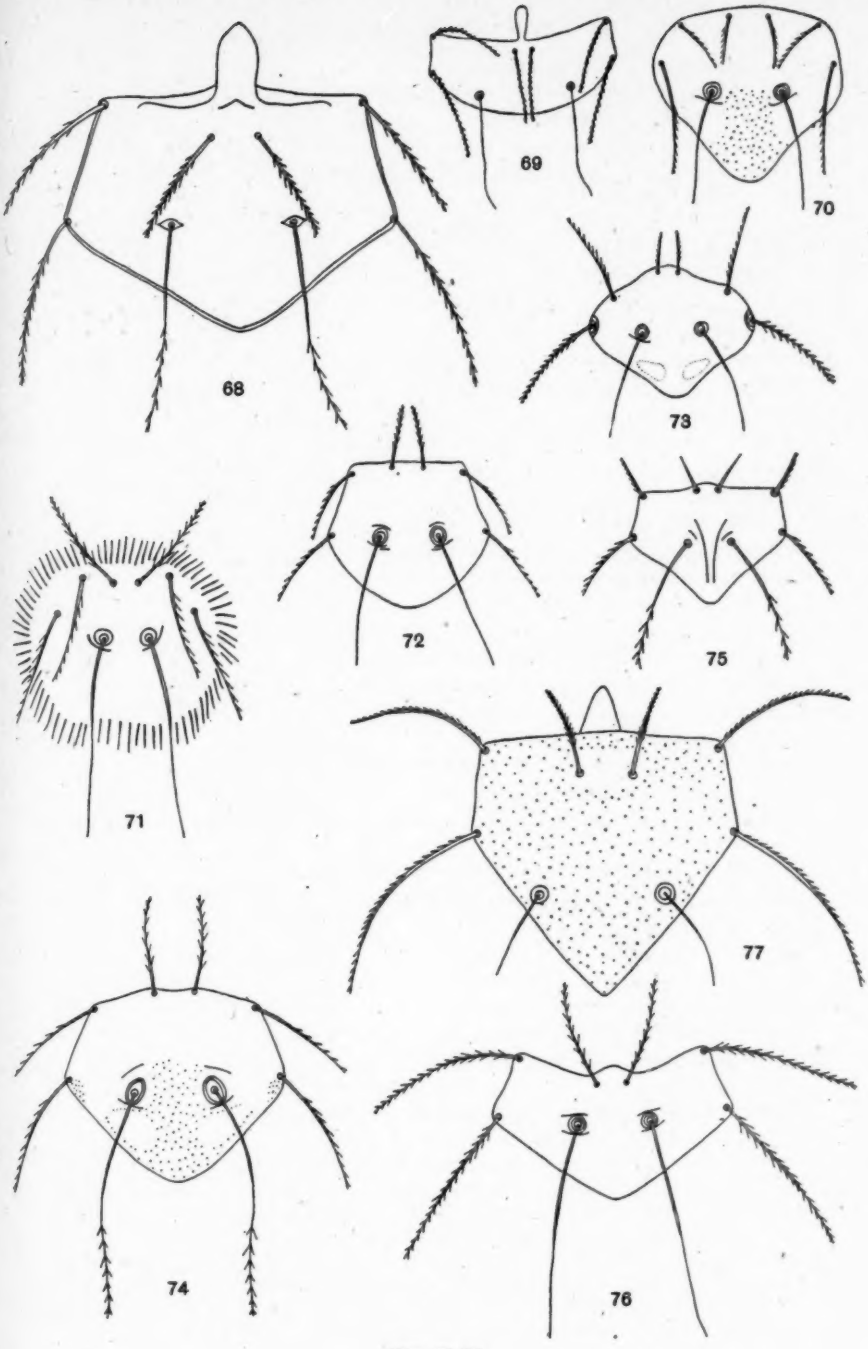
Neoschöngastia signator Ewing, 1931 (Fig. 80).
Host. Wood rat.
Locality. Wilburton, Okla.

Neoschöngastia scelopori Ewing, 1931.
Host. Spiny-fence lizard (*Sceloporus spinosus* Wiegmann).
Locality. Uvalde, Texas.

Neoschöngastia brevipes Ewing, 1931 (Fig. 81).
Host. Northern white-footed mouse (*Peromyscus leucopus noveboracensis* Fischer).
Locality. College Park, Maryland.

Legends to figs. 68–77

68. *Leeuwenhoekia australiensis* Hirst. 69. *Leeuwenhoekia gateri* n.sp. 70. *Hannemania dunni* Sambon. 71. *Hannemania edwardsi* Sambon. 72. *Hannemania eltoni* Sambon. 73. *Hannemania newsteadi* Sambon. 74. *Hannemania stephensi* Sambon. 75. *Hannemania hylodeus* Oudemans. 76. *Hannemania hirsuta* Ewing. 77. *Hannemania hepatica* Fonseca.



Figs. 68–77 .

Neoschöngastia sciuricola Ewing, 1925.
Host. Richardson's red squirrel (*Sciurus hudsonicus richardsonii* Bachman).
Locality. Florence, Montana.

Neoschöngastia trouessarti Oudemans, 1910.
Host. Naked-tailed opossum (*Metachirus opossum* Seba.).
Locality. South Brazil.

Neoschöngastia oudemansi Walch, 1923 (Fig. 82).
Host. Rat.
Locality. Deli, Sumatra.

Neoschöngastia muris Walch, 1923 (Fig. 83).
Host. Rat.
Locality. Deli, Sumatra.

Neoschöngastia gallinarum Hatori, 1920 (Fig. 84).
Host. Fowl (*Gallus gallus domesticus* Briss.).
Locality. Formosa.

Neoschöngastia schuffneri Walch, 1923 (Fig. 85).
Host. Man.
Locality. Deli, Sumatra.

Neoschöngastia brasiliensis Fonseca, 1935 (Fig. 86).
Host. Rat no. 334 (undetermined).
Locality. Butantan, Sao Paulo, Brazil.

Neoschöngastia dasyproctae Ewing, 1937.
Host. Aguti (*Dasyprocta variegata* Tschudi).
Locality. Muzo, Boyaca Department, Colombia.

Neoschöngastia yeomansi Gunther, 1939 (Fig. 87).
Host. Bush fowl.
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Neoschöngastia impar Gunther, 1939 (Fig. 88).
Host. Bandicoots, Monkton's melomys, Stalker's melomys, Rufous' melomys, Brown bush rat and *Rattus ringens.*
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

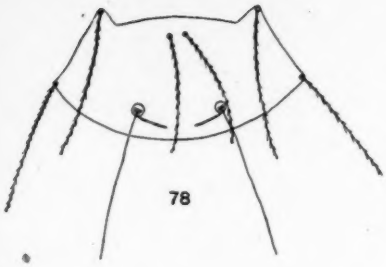
Neoschöngastia edwardsi Gunther, 1939 (Fig. 89).
Host. Bush fowl, bandicoot.
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Neoschöngastia lorius Gunther, 1939 (Fig. 90).
Host. Parrot (*Lorius voratus* subsp.).
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Neoschöngastia backhousi Gunther, 1939 (Fig. 91).
Host. Bush fowl.
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Legends to figs. 78–92

78. *Hannemania ochotrona* n.sp. 79. *Neoschöngastia americana* Hirst. 80. *Neoschöngastia signator* Ewing. 81. *Neoschöngastia brevipes* Ewing. 82. *Neoschöngastia oudemansi* Walch. 83. *Neoschöngastia muris* Walch. 84. *Neoschöngastia gallinarum* Hatori. 85. *Neoschöngastia schuffneri* Walch. 86. *Neoschöngastia brasiliensis* Fonseca. 87. *Neoschöngastia yeomansi* Gunther. 88. *Neoschöngastia impar* Gunther. 89. *Neoschöngastia edwardsi* Gunther. 90. *Neoschöngastia lorius* Gunther. 91. *Neoschöngastia backhousi* Gunther. 92. *Neoschöngastia dubia* Gunther.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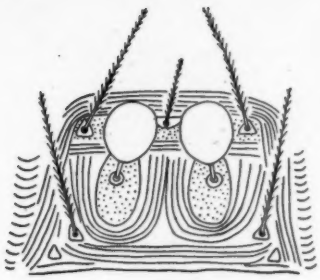
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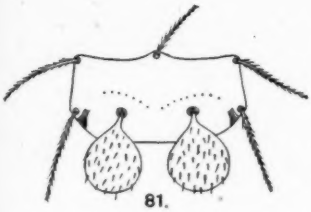
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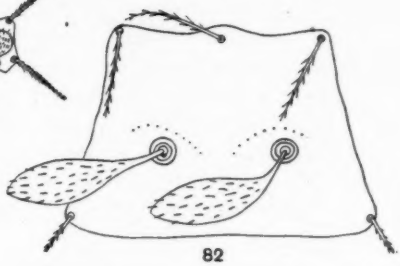
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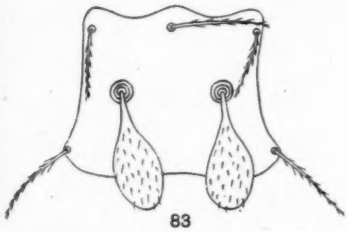
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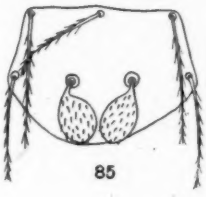
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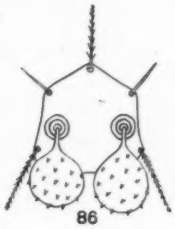
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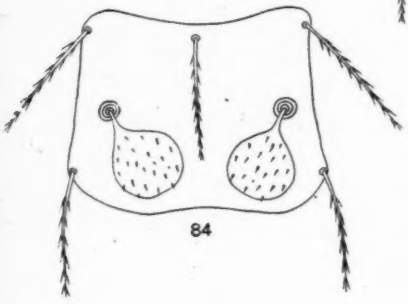
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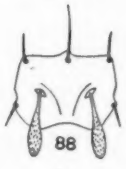
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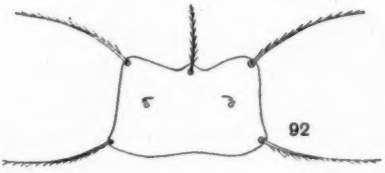

92

Figs. 78–92

Neoschöngastia dubia Gunther, 1939 (Fig. 92).
Host. Bush fowl.
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Neoschöngastia retrocincta Gunther, 1939 (Fig. 93).
Host. Bush fowl.
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Neoschöngastia kallipygos Gunther, 1939 (Fig. 94).
Host. Arboreal mouse (*Melomys* sp.), bandicoot, Brown's rat, etc., etc.
Locality. Lower Bulolo River Basin, Morobe, New Guinea.

Neoschöngastia lacunosa Gater, 1932 (Fig. 95).
Host. Large spiny-backed rat (*Rattus sabanus vociferans* Miller).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Neoschöngastia malayensis Gater, 1932 (Fig. 96).
Host. Malay grey rat (*Rattus canus malaisia* Kloss).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Neoschöngastia debilis Gater, 1932 (Fig. 97).
Host. Pencil-tailed rat (*Rattus cremoriventer cremoriventer* Miller).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Neoschöngastia mutabilis Gater, 1932.
Host. Large spiny-backed rat (*Rattus sabanus vociferans* Miller).
Locality. Sungei Buloh, Selangor, Federated Malay States.

Neoschöngastia indica Hirst, 1915 (Fig. 98).
Host. Indian mole-rat (*Gunomys bengalensis* Gray).
Locality. Calcutta, India.

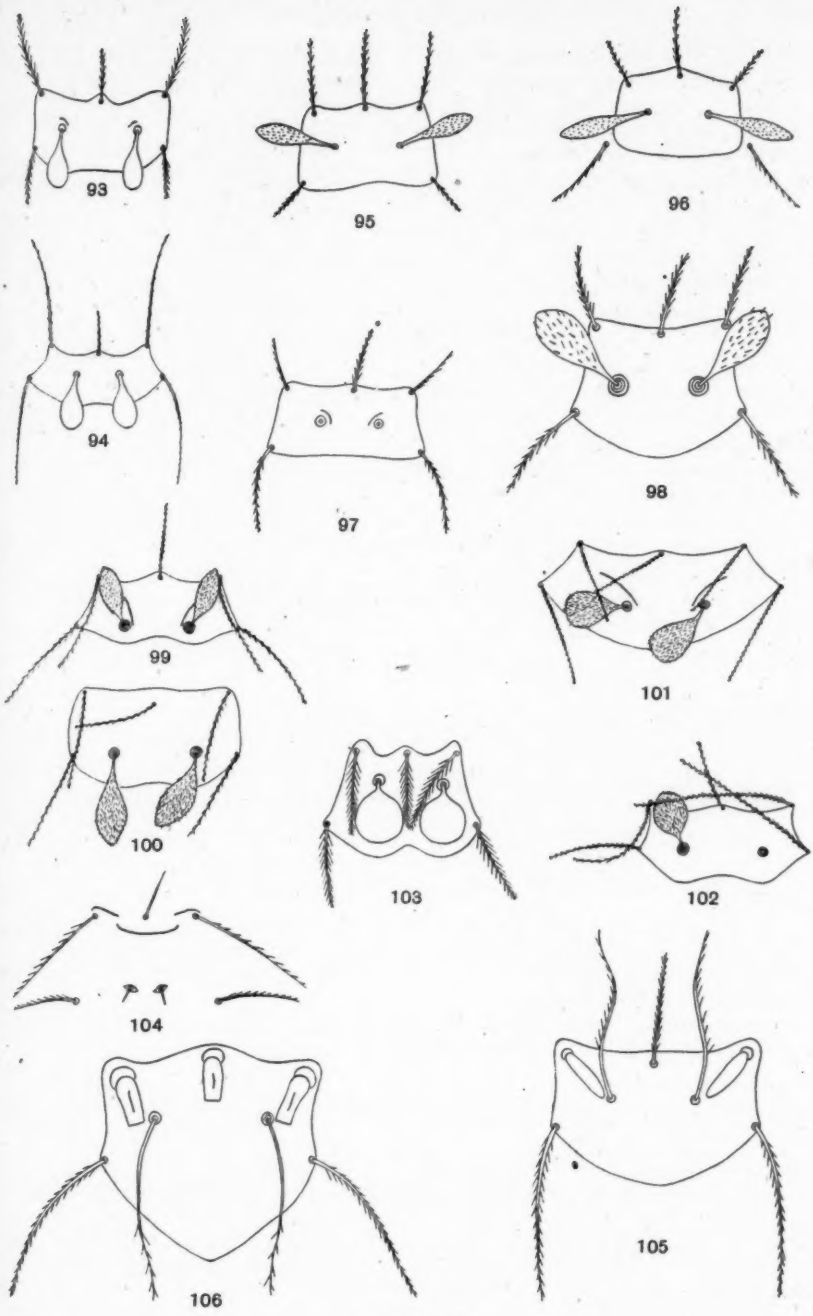
Neoschöngastia samboni n.sp. (Fig. 99).
Host. Pika.
Locality. Rassis Hole, Montana, 2 December 1933.
Dorsal setae about 46, arranged in rows of 12, 12, 10, 8, 4.
Measurements: 0·37 × 0·22 mm.

Neoschöngastia otomyia n.sp. (Fig. 100).
Host. Vlei or Swamp rat (*Otomys tugelensis pretoriae* Roberts).
Locality. Onderstepoort, South Africa, 23 May 1939.
Dorsal setae about 26, arranged in rows of 10, 6, 6, 4.
Measurements: 0·21 × 0·17 mm.

Neoschöngastia guntheri n.sp. (Fig. 101).
Host. Cony.
Locality. Antonito, Colorado, 9 September 1931.
Dorsal setae about 110–120.
Measurements: 0·61 × 0·52 mm.

Legends to figs. 93–106

93. *Neoschöngastia retrocincta* Gunther. 94. *Neoschöngastia kallipygos* Gunther. 95. *Neoschöngastia lacunosa* Gater. 96. *Neoschöngastia malayensis* Gater. 97. *Neoschöngastia debilis* Gater. 98. *Neoschöngastia indica* Hirst. 99. *Neoschöngastia samboni* n.sp. 100. *Neoschöngastia otomyia* n.sp. 101. *Neoschöngastia guntheri* n.sp. 102. *Neoschöngastia aethomyia* n.sp. 103. *Neoschöngastia mirafra* n.sp. 104. *Endotrombicula penetrans* Ewing. 105. *Fonsecia ewingi* Fonseca. 106. *Fonsecia travassosi* Fonseca.



Figs. 93–106

Neoschöngastia aethomyia n.sp. (Fig. 102).
Host. Graham's Rock mouse (*Aethomys namaquensis grahami* Roberts).
Locality. Caxton, Bathurst, South Africa, 14 May 1939.
Dorsal setae about 32, arranged in rows of 6, 6, 6, 6, 4, 4.
Measurements: 0·44 × 0·32 mm.

Neoschöngastia mirafra n.sp. (Fig. 103).
Host. Lark (*Mirafra africana tropicalis*).
Locality. Katwe, Toro, Uganda, 14 July 1939.
Dorsal setae about 28, arranged in rows of 6, 6, 6, 6,-4.
Measurements: 0·4 × 0·3 mm.

Genus **Odontacarus** Ewing, 1929

Odontacarus australis Ewing, 1929.
Host. Lesson's peruvian lizard (*Tropiduras peruvianus* Lesson).
Locality. Verrugas Cañon, Lima, Peru.

Odontacarus dentatus Ewing, 1925.
Host. Sonora white-tailed deer (*Odocoileus couesi* Coues & Yarrow).
Locality. Sonora, Texas.

Genus **Endotrombicula** Ewing, 1931

Endotrombicula penetrans Ewing, 1931 (Fig. 104).
Host. Smith's pigmy frog (*Arthroleptis minutus* Blgr.).
Locality. Mount Sagalla, Kenya.

Genus **Fonsecia** n.g.

Fonsecia ewingi Fonseca, 1932 (Fig. 105).
Host. Merrem's Boipeva snake (*Ophis merremii* Wagler).
Locality. Matto Grosso, Brazil.

Fonsecia travassosi Fonseca, 1935 (Fig. 106).
Host. Cainana rat snake (*Spilotes pullatus* L.).
Locality. Angra dos Reis, Rio de Janeiro.

APPENDIX

A key to the genera of Trombiculinae, based on larva characters.
[According to H. E. Ewing (1938).]

A. Dorsal plate with a median seta on its anterior margin.
  B. Pseudostigmatic organs strongly clavate or capitate.
    C. Anterolateral setae of dorsal plate large, barbed, similar to the posterolateral setae.
      D. Each chelicera with a row of dorsal teeth; palpal claw usually with two prongs. **Schöngastia** Oudemans.
      DD. Each chelicera with not more than one dorsal tooth.
        E. Palpal claw with two or three prongs. **Neoschöngastia** Ewing.
        EE. Palpal claw with five or seven prongs, the accessory prongs being in two or three pairs; dorsal tooth of chelicera vestigial or absent. **Euschöngastia** new genus.
    CC. Anterolateral setae of dorsal plate minute, simple. Parasitic on bats. **Doloisia** Oudemans.

BB. Pseudostigmatic organs setiform or flagelliform, frequently with barbs.

C. Each chelicera with a row of dorsal teeth; ventral tooth sometimes absent.

D. More than three subequal dorsal teeth on each chelicera; dorsal plate well developed; median seta pectinate. **Odontacarus** Ewing.

DD. Three unequal dorsal teeth on each chelicera; dorsal plate vestigial; median seta simple. Living under surface of skin of amphibians. **Endotrombicula** Ewing.

CC. Each chelicera with a single dorsal tooth; ventral tooth present.

D. Palpal claw typically divided into three prongs, dorsal abdominal setae usually more than 30. **Trombicula** Berlese.

DD. Palpal claw divided into two prongs; dorsal abdominal setae usually less than 30. **Eutrombicula** new genus.

AA. Dorsal plate without median seta.

B. Dorsal plate with a pair of submedian setae on anterior margin.

C. Dorsal plate with an anterior median process but without crista. **Leeuwenhoekia** Oudemans.

CC. Dorsal plate without anterior median process but frequently with crista; each chelicera obliquely flattened at distal end, forming a 'spearhead' with teeth on its lateral margin. **Hannemania** Oudemans.

BB. Dorsal plate without a pair of submedian setae on anterior margin.

C. Each tarsus armed with two unequal claws; pseudostigmatic organs represented by a pair of simple setae; all setae both on body and appendages, simple. **Hemitrombicula** new genus.

CC. Each tarsus armed with three claws, the middle one usually more slender and longer than the others; pseudostigmatic organs clavate or capitate.

D. All setae on dorsal plate (exclusive of pseudostigmatic organs) marginal.

E. Dorsal plate with four or more pairs of lateral setae. **Gahrliepia** Oudemans.

EE. Dorsal plate with less than four pairs of lateral setae.

F. Dorsal plate with three pairs of lateral setae, eyes present. **Schöngastiella** Hirst.

FF. Dorsal plate with two pairs of lateral setae; eyes absent. **Walchia** Ewing.

DD. Some of setae on dorsal plate not marginal; eyes usually present. **Gateria** new genus.

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Figs. 3, 26, 46, 76, 80, 81 and 104 from *Proc. U.S. nat. Mus.* 1931, **80**, 8.
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Figs. 6, 12, 13 and 85 from *Kitasato Arch.* 1923, **5**.

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MORPHOLOGICAL VARIATION IN *PLASMODIUM VIVAX* GRASSI & FELETTI, 1890

By J. W. FIELD

From the Institute for Medical Research, Federated Malay States

(With Plate I)

THE parasites of malaria in common with all living organisms sometimes appear in abnormal forms. Within each species there is a small range of variation. The shape, the size, the number of merozoites, the changes produced in the host red cells and other characters are not morphological constants; and though variation is usually within the limits of the conventional schematic descriptions, occasional variant forms may show a wide departure from the normal appearances, so wide at times that the specific identity may be in doubt.

Within the past year two parasites believed to be abnormal forms of *P. vivax* have been observed in this Institute. The first showed an extraordinary tendency to multiple infection of the host cells; the second, an unusually small size at maturity. The aberrant appearances are here briefly described.

1. MULTIPLE INVASION OF SINGLE RED CELLS

Loke Chong, an elderly Cantonese, was admitted to the General Hospital, Kuala Lumpur, on 1 February 1940 and referred to the Malaria Research Division of this Institute on the following day with acute *vivax* malaria. He had had fever at home for 5 days and had taken a little quinine but he did not know how much. He could not recall previous fever within 12 months. Blood films taken on the morning of 2 February, the 7th day of the attack, revealed a very heavy *vivax* infection. Quinine treatment was given for 8 days and the patient made an uneventful recovery.

Clinically the case was unusual in the low fever associated with exceptionally heavy infection. The parasite count was over 100,000 per cu. mm. of blood, yet at no time in hospital did the temperature rise to 100° F. The most surprising feature, however, was the parasitological picture revealed in the blood films taken in the morning of 2 February. All stages of *P. vivax* were present but mainly young trophozoites. Advanced trophozoites, schizonts and gametocytes were morphologically typical, but the young trophozoites showed an extraordinary tendency to crowd into particular cells. Many cells contained four, five, and even six parasites; one cell appeared to have eight. The number of young trophozoites in a successive 500 recently invaded cells is shown in Table 1.

Pl. I, figs. 1–12, illustrates typical appearances. Eight hours later the tendency to multiple infection of cells was much less; within 24 hr. it had gone. Subsequent films disclosed no apparent abnormality.

The presence of more than two parasites in a single cell, though common with *P. falciparum*, is unusual with other species; more than four *vivax* parasites per cell is rare. The phenomenon in the present case was associated with exceptionally heavy infection in an old man who showed little febrile reaction.

Table 1. *Multiple infections in* 500 *cells containing young* vivax *trophozoites*

	Parasites per cell						
	1	2	3	4	5	6	6+
Infected cells	312	99	40	35	9	4	1
Percentage	62	20	8	7	2	1	—

A case somewhat similar is reported by Chalmers & Archibald (1920); while Wenyon records having seen six *vivax* rings in a single cell. Chalmers & Archibald's case was a British soldier in Khartoum with a heavy untreated *vivax* infection. Many cells contained from three to six young trophozoites; one cell contained seven. The colour drawings accompanying their paper show appearances which correspond closely with those seen in the case now described.

The biological explanation of the phenomenon may have been:

(*a*) that certain red cells are peculiarly attractive to merozoites;

(*b*) that 'rings' may divide by simple fission;

(*c*) that red cells may occasionally be subjected to massive invasion by groups of incompletely separated merozoites.

(*a*) There is evidence that *P. vivax* tends to select red cells which have not yet attained maturity. Hegner (1938) and others have shown that reticulocytes are invaded more often than mature red cells: in one case observed by Hegner the relative frequency of invasion of reticulocytes and mature cells, had young and mature red cells been equally available, would have been over 1000 to 1. When, as in the case now described, half a dozen parasites are crowded into a single cell while neighbouring cells are uninfected, it is evident that the occurrence is no mere chance. There is an important objection, however, to the assumption that the phenomenon is due to a selective invasion of immature cells: multiple infection of single cells is much more common with *P. falciparum* than with *P. vivax*, yet there is reason to believe that it is *P. vivax* which has the greater tendency to a selective attack on cells which are immature.

(*b*) Many observers have suggested that malarial parasites at the ring stage may occasionally divide by simple fission. Beach (1936), for example, has observed appearances in *P. vivax* which he interprets as indicating that ring forms may divide to produce two independent parasites. Hingst (1934) believes that *P. falciparum* may sometimes divide by binary fusion. 'How else', he writes, 'can a double nucleated parasite be explained, one with two organelles performing the same function, that of reproduction? Where else in biology do we find such a duplication? How else can we give a logical explana-

tion to the multiple invasion of red cells that is so characteristic of *P. falciparum*?' Chalmers & Archibald (1920) apparently held a similar view. They point out that although the usual method of asexual reproduction in the telesporidia is by the formation of merozoites—and human malarial parasites are no exception to the general rule—simple and multiple fission are common in the flagellata and simple fission is noted in the piroplasmidae. Simple and multiple fission, they believe, may also occur exceptionally with malarial parasites; the whole process being a throw-back to a method of reproduction useful perhaps to some remote ancestor.

(*c*) A third possible explanation is that exceptionally cells may be invaded by entire groups of incompletely separated merozoites. Incomplete separation of merozoites is seen not infrequently in lysed blood films, and there appears no obvious reason why, with the natural bursting of infected cells, every merozoite should necessarily separate completely from its neighbours, or why conjoined merozoites should not jointly invade a fresh cell.

2. Mature schizonts of small size and reduced number of merozoites

Karupannan, an Indian infant born on a malarial plantation in Selangor, one of the Federated Malay States, developed a malarial attack on 3 March 1939, when 16 days old. The mother had had *vivax* malaria 2 months before the birth of the child, but had no fever at the time of delivery. The child's infection may have been congenital but may equally well have been acquired after birth, as there was active *vivax* transmission on the plantation.

The attack ran a normal course and responded well to quinine. Blood films taken before treatment were sent to the laboratory by the courtesy of Dr J. F. Mitchell, the plantation medical officer. They revealed a very heavy infection with a *vivax*-like parasite at all stages of asexual development (Pl. I, figs. 13–24). The infected cells were enlarged and heavily stippled with Schüffner's dots, but the parasites themselves differed from normal *vivax* in the small size of the mature schizonts and the reduced number of merozoites. Most of the fully segmented parasites occupied no more than about two-thirds of the host cells, though some were of normal size. The merozoite count in 100 schizonts apparently fully segmented is tabulated below and compared with that of a typical *vivax* infection chosen at random.

Table 2. *Number of merozoites in apparently mature schizonts*

	Number of merozoites															
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	20+
Present case	3	2	10	15	21	16	15	10	5	3	0	0	0	0	0	0
Typical *vivax* chosen at random	0	0	0	0	0	0	4	3	14	15	30	13	12	5	3	1

Other abnormal features were (*a*) an unusual number of multiple-infected cells, (*b*) a tendency for the infected cells to assume bizarre fimbriated outlines,

(*c*) a tendency to a compact form, and (*d*) a lesser degree of enlargement of infected cells than is usual with *P. vivax*.

The gametocytes were indistinguishable in size and appearance from those of *P. vivax*.

There was a recurrence of fever and parasites were again found in the peripheral blood on 1 June, 3 months later. The only parasites now seen were young trophozoites and gametocytes which could not be distinguished from *P. vivax*. Early quinine treatment was indicated on clinical grounds, and there was then little opportunity of deciding whether the abnormal schizonts found in the primary attack had reverted to type.

A similar *vivax* variant has been described by Das Gupta (1939) in a congenital infection. A film taken from an infant 15 hr. old revealed a heavy infection with a *vivax*-like parasite which closely resembled that now described.

It is perhaps significant that in both of these cases the infection was either congenital or early post-genital, and it seems possible that the morphological aberration may have been related to the growth of the parasite in an infant host.

COMMENTS

The occurrence of abnormal forms of *P. vivax* is well known. They may be found in small numbers in a single film; they may persist throughout an infection; they may even survive interhuman passage: but as a rule they seem to occur under conditions which suggest some change in the conditions of life of the parasite. Thus Giovannola (1935) found that the Wagner-Jauregg strain of *P. vivax* which was isolated in the Balkans in 1919 and subsequently maintained for 15 years by interhuman passage had changed in the meantime. There was less enlargement of the host cell, the parasites tended to assume a globular and compact shape, gametocytes were fewer, the chromatin divided earlier, the schizonts were smaller and the merozoites were reduced in number. Kühnhold (1926), in a strain of *P. vivax* which appeared normal until the third human passage, observed that the schizogonic stage was modified in the fourth passage and produced only from six to ten merozoites. Plehn (1925) observed premature segmentation in a *vivax* strain used for the treatment of general paralysis; Friedmann (1937), in a comparison of three *vivax* strains also used for malario-therapy, noted abnormal forms, some of which resembled *P. ovale*; while Hauer (1937) observed that a strain of *P. ovale* from the Hamburg Institute of Tropical Diseases produced *vivax*-like forms after the 23rd human passage, but by the 38th passage had again reverted to type. Similarly, Missiroli & Siniscalchi (1929), in a study of a *vivax* strain from the Pontine Marshes, observed, among other changes, a premature division of the chromatin with the formation of eight to twelve merozoites only. The special conditions of direct passage from man to man, they believe, tend to modify the schizogonic phase of the parasite and to produce premature division of the chromatin.

Changes in the form of *P. vivax* observed in the course of malario-therapy are associated with, and apparently due to the altered conditions of life

resulting from direct passage from man to man and the elimination of the mosquito cycle; similar changes may, it seems, also occur in chronic *vivax* infections acquired naturally. James (1920), for example, in old-standing cases of *vivax* malaria contracted during the Great War, found unusually small schizonts in cells but slightly enlarged: while Giovannola (1935) is of opinion that a reduction in the size of schizonts and in the number of merozoites is not unusual in chronic infection. 'The parasites', he states, 'are obliged to repeat their asexual cycle without passing through mosquitoes and they seem to adapt their biology to this special condition.'

The two parasites now described were found under conditions which suggest a departure from the normal parasite-host relationship. The multiple infected cells in case 1 were from an old man with heavy infection and little clinical response: the parasites were apparently growing in an environment more than usually favourable to schizogonic multiplication. Case 2 was an infant 16 days old: transmission of the infection directly from the mother is a clear possibility; similar aberration in form, we may recall, was found by Das Gupta in an infant a few hours old.

The evidence that changes in the conditions of life of *P. vivax* may alter the morphology emphasizes the necessity for caution in the creation of new species and varieties. Disturbance of the normal alternation of life cycles or an abnormal reaction on the part of the host may apparently induce transient changes in form; and aberration—in the size and shape of the infected cell, in the size, shape and texture of the parasite, in the time of chromatin division or the number of merozoites—unless consistent, is thus an insufficient indication of distinctive specific identity. New specific names, as Wenyon (1926) has stressed, are justified only when it can be shown that the changes are constant. They are valid only when the abnormal characters remain for generation after generation while the parasite follows its normal way of life.

Summary

Two *vivax*-like parasites, believed to be true *P. vivax* but in abnormal form, are described. The deviation from normal appearances was probably due, it is suggested, to growth in an abnormal environment.

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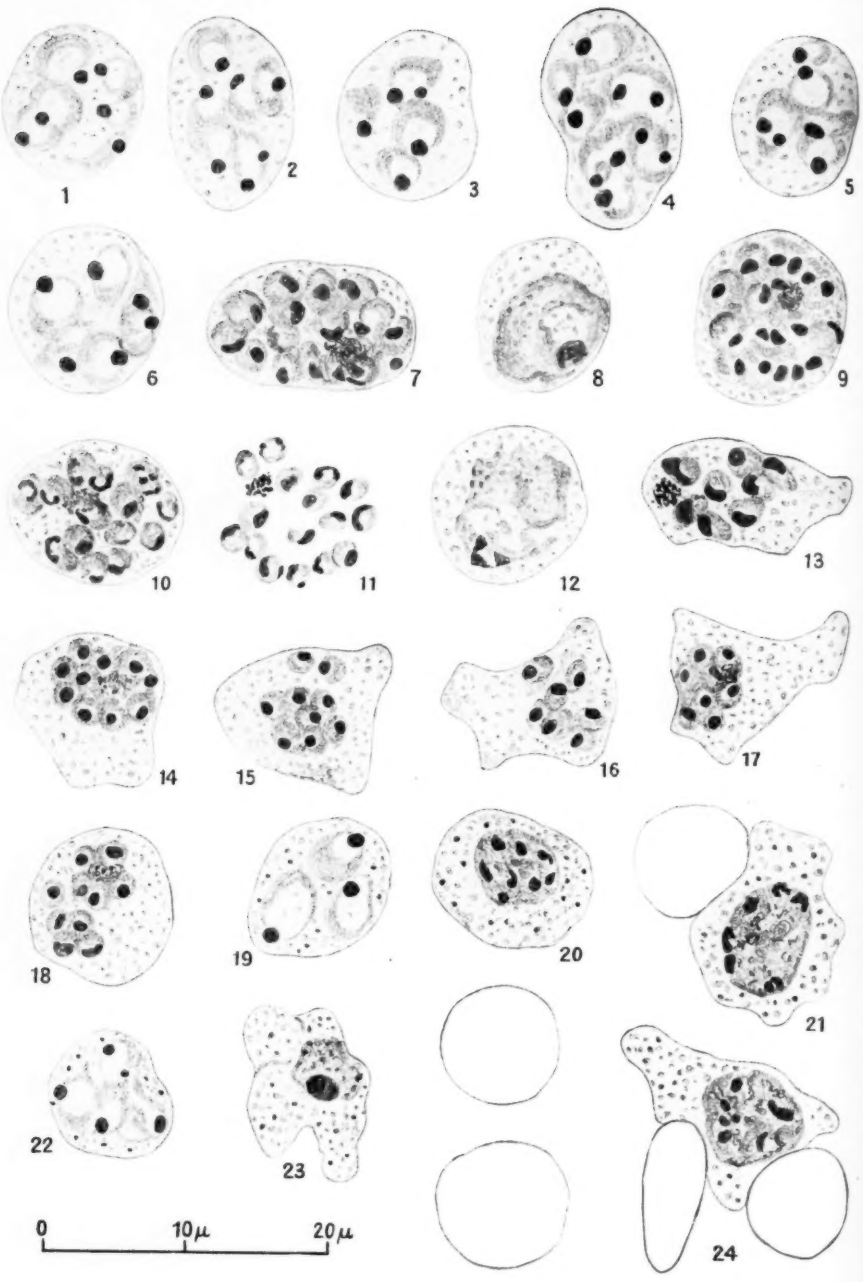

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EXPLANATION OF PLATE I

Figs. 1–12. Camera lucida drawings made from a blood film taken from an elderly Chinese male on the seventh day of a primary attack of *vivax* malaria.

Clinically the case was unusual in the small febrile reaction to heavy infection; parasitologically in the extraordinary tendency for the 'ring' forms to crowd into particular cells. Figs. 1–6 illustrate this tendency. Figs. 7–12 show the stage of development of most of the associated forms—well-grown trophozoites with many advanced and mature schizonts.

Multiple invasion of single cells was most marked while the peripheral blood contained many ripe segmenters: 24 hr. later the phenomenon had passed and was not seen again.

Figs. 13–24. Camera lucida drawings made from blood films taken from an infant 16 days old. The mother had had *vivax* malaria 2 months before delivery but whether the infection of the infant was congenital or acquired was not known.

The main departure from normal *P. vivax* is shown in Figs. 13–18. The schizonts are small, sometimes occupying less than one-half of the host cell, and the number of merozoites in ripe segmenters is reduced: some of them resemble *P. malariae* except for their presence in enlarged cells with typical *vivax* stippling.

Other abnormal features are the tendencies: (*a*) to multiple invasion of single cells (Figs. 19, 22); (*b*) to compact circular or ovoid form (Figs. 20, 21, 23, 24); (*c*) to distortion of the host cell (Figs. 13, 16, 17, 21, 23, 24), particularly where infected cells impinge on other cells (Figs. 21, 24); and (*d*) to lesser enlargement of infected cells than is usual with *P. vivax*.

The clear circles are outlines of normal erythrocytes to indicate the degree of enlargement of the parasitised corpuscles.

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ON *BRAULA COECA* NITSCH AND ITS AFFINITIES

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(With 8 Figures in the Text)

For over one hundred years *Braula coeca* has been classified among the Diptera Pupipara to which group it was originally relegated by Nitsch (1818). It was not until 1921 that Skaife showed that the insect is oviparous. This author confined himself to a brief general account of the life history together with short descriptions of the egg and larva. Subsequently further accounts of the biology and metamorphoses of this insect have been given by various writers including Arnhart (1923), Phillips (1925), Herrod-Hempsall (1931), Suire (1931), Örösi-Pal (1938), Hennig (1938*a*) and Toumanoff (1939). The most complete article is that by Hennig, but none of these writers discusses the morphological aspect sufficiently adequately to enable any conclusions to be drawn regarding the taxonomic position of the Braulidae. An accurate account of the larva is consequently needed and especially an investigation of the pseudocephalon, the bucco-pharyngeal armature and the tracheal system. The present paper is intended as a contribution towards achieving this object. The structure of the adult *Braula* is dealt with to a considerable extent in the admirable memoir by Massonnat (1909) while Müggenburg (1892), Börner (1908), Alfonsus & Braun (1931) and Hennig (1938) add some further details.

Material and technique

Beehives were searched for eggs and larvae of *Braula* in the Cambridge district during several years without result. It has since become evident that the species is abundant during certain seasons only, becoming scarce or absent in the intervening periods. Material in all stages was obtained during the months of July to November: pupae were only found in October and November, while during the winter and early spring only adults were obtained. In order to secure material of different stages, from the egg to the pupa, infested cells were decapped by means of a sharp knife and the cappings placed near to a beehive. As stated by Herrod-Hempsall the bees quickly removed any adhering honey and left the wax tunnels of the *Braula* completely cleaned. The cappings were then placed inner side upwards on the stage of a binocular microscope and the tunnels were slit open by means of a very fine needle in order to secure the larvae and pupae of *Braula*. Removal of the wax by means of solvents was also tried but was much less satisfactory. For preparations of the mouthparts and spiracles the parts concerned were placed in 1% solution of commercial pepsin in distilled water. To 100 c.c. of this fluid 1 c.c. of concentrated hydrochloric acid was added. The container, with the parts to be digested, was then

placed in a water oven kept at 30° C. until the desired condition was attained. This method is safer and less drastic for minute delicate objects than treatment with the more usual solution of potassium hydroxide.

Habitat and behaviour of *Braula*

The eggs were found on the inner or lower side of the cappings of honey cells and occasionally on the wall of the cells themselves. The whole development takes place normally beneath the cappings of the honey cells and not among the brood cells. The young larva constructs a tunnel by gnawing the inner surface of the wax capping and moulds the fragments thus separated to form a tube or tunnel within which it lives. It moves with facility either backwards or forwards in this tunnel. The latter is thread-like when first formed but

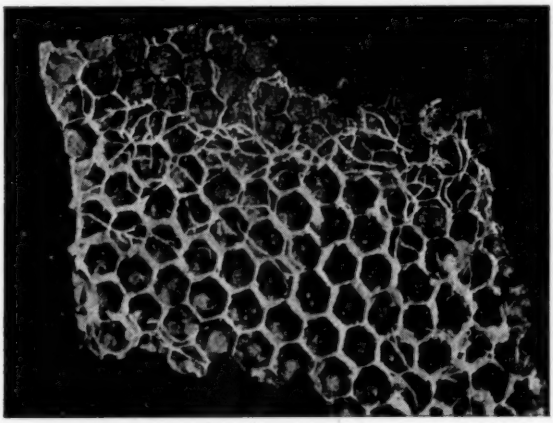

Fig. 1. Photograph of cappings of honey cells taken from the inner side. The presence of larvae of *Braula* is indicated by the tubular burrows passing from one cell to another. Natural size.

assumes a larger calibre as the larva grows older and may traverse several cells on its course. In a badly infested hive the tunnellings may form a rather complex network of whitish tubes which are the work of many larvae. Although best seen from the inner side of the cappings as shown in Fig. 1 they were also visible through the caps of closed cells when viewed externally. Consequently, only very little experience is needed to detect the existence of *Braula* in a beehive by the presence of such tunnellings. While Skaife (1921) states that the larvae are found in the brood cells where they also pupate, beside the pupae of the bees, this has not been the experience of subsequent observers. Arnhart (1923) was the first to note that development occurs on the inner side of the cappings of the honey cells as described above.

The digestive system of the larva contains wax and often pollen grains also. Some account of its anatomy and histology is given by Örösi-Pal (1938), who

states that the epithelial cells of the mid-intestine contain micro-organisms. This author suggests that the micro-organisms are concerned in the digestion of wax, on the analogy of Dickman's work (1933) on nutrition of the larva of the wax moth (*Galleria mellonella*).

Pupation takes place in the larval tunnel, and prior to metamorphosis the larva apparently cuts a very fine circular groove to form a line of weakness on the inner wall of the pupal chamber (Herrod-Hempsall, 1931). When the imago issues from the pupa its inflated ptilinum forces a circular lid or operculum outwards and the insect makes its exit through the hole thus formed.

After eclosion the adult *Braula* makes its way to the bees and comes to reside on the body of usually a queen or worker. Out of forty-eight infested bees that were collected near Cambridge one was a drone and the remainder workers. In three instances two examples of *Braula* were found on a single worker bee: in the other cases the occurrence was singly on each bee, and the most usual position is at the constriction between the thorax and abdomen. Fourteen examples were counted on a single queen on one occasion. The number recorded from individual queen bees is often surprisingly large and there seems little doubt, as described by Lósy (1902), that *Braula* will migrate from the workers to the queen. Thus Marboud (1907) states that thirty-one *Braula* were removed from a queen bee: the next day thirty-three more were taken off the same individual bee, and the process was continued at intervals until finally 371 examples were obtained from off the one insect. Hammar (1858), quoted by Phillips (1925), reports taking 187 *Braula* from a single queen and that sixty-four more examples were collected off the same bee. The accuracy of this statement is questioned by Timm (1917) on account of the relatively large size of *Braula* as compared with that of the bee. It would appear impossible for a queen to carry a population as numerous as that quoted, and this author suggests that mites or triungulin larvae of *Meloë* were mistaken for *Braula*.

As regards its habits the adult *Braula* is now generally regarded to be an inquiline in the hive. Its mouthparts, it may be added, are incapable of piercing the integument of the bee. A detailed account in Hungarian of the method of feeding by *Braula* has been given by Lósy (1902); an abstract by Gorka makes available what appear to be the essentials of the process. A free translation of Gorka's review is given by Phillips (1925) to which the reader is referred. Very briefly it may be stated that the *Braula* migrates to the head of the bee when about to feed and takes up a position on the open mandibles and labrum. Its mouthparts reach into the cavity at the base of the extended glossa of the bee, near to the opening of the duct from the salivary glands. Because of the irritation caused by the unusual penetrating body saliva, according to Lósy, is discharged reflexly, which the *Braula* sucks up. His account seems to imply that salivary secretion forms the only food, but the matter requires further investigation. Phillips, it may be added, made smears from the contents of the alimentary canal but was unable to identify the liquid diet.

The egg (Fig. 2)

The egg appears white to the unaided eye. The chorion has a closely punctured surface (Fig. 2 B) and an exochorion is developed as two lateral flanges. Since these flanges are extremely fragile considerable variation results if they are included when making measurements of the egg. Each flange is flat and extends parallel to its fellow and to the long axis of the egg. The surface shows a coarsely reticulated pattern (Fig. 2 C) and the margin is irregular. This kind of egg resembles that termed the *Phaonia* type by Muirhead Thomson (1937) more closely than any other among Diptera. Besides *Phaonia variegata* this author states that he has only found it in *Muscina pabulorum* and *Graphomyia maculata*, all three species being Anthomyidae. His suggestion that the flanges may possibly serve either for attachment to a solid substratum, or for supporting the egg right way up in a very fluid medium, seems applicable to *Braula* also, when submersion in honey would most likely prove fatal to the larva.

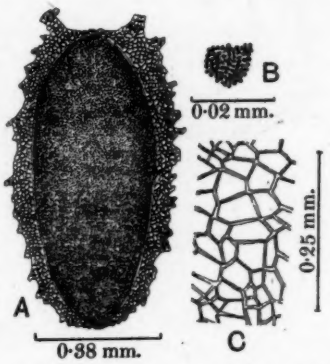

Fig. 2. A. Egg of *Braula*. B. Portion of chorion. C. Portion of lateral flange showing reticulated surface.

The dimensions of the egg were measured immediately after mounting in De Faure's medium. The average length, without including the flanges, ranged from 0·78 to 0·81 mm. and the breadth from 0·28 to 0·33 mm. Including the flanges one very typical egg measured 0·84 × 0·42 mm.

The larva

The larva passes through three instars as is usual among the Cyclorrhapha. When newly issued from the egg its average size is 0·8 mm. long and 0·25 mm. in diameter. In the third instar, when fully grown, its average length is 2·25 mm. with a diameter of 0·5 mm. The general form of the larva differs very little in successive instars. The body is composed of three thoracic and eight abdominal segments together with a much reduced pseudocephalon (Figs. 3, 4).

The cuticle over the general surface of the body appears granulated under a low magnification and this is due to the presence of tubercles of varying sizes. In sections stained with Heidenhain's iron-alum haematoxylin these tubercles become heavily coloured while the remainder of the cuticle remains unstained (Figs. 5, 7).

The most characteristic external feature is the presence of whorls or girdles of sensoria on the first four segments and also on the last segment (Fig. 3). The sensoria are largest over the dorsal surface becoming smaller ventralwards. On

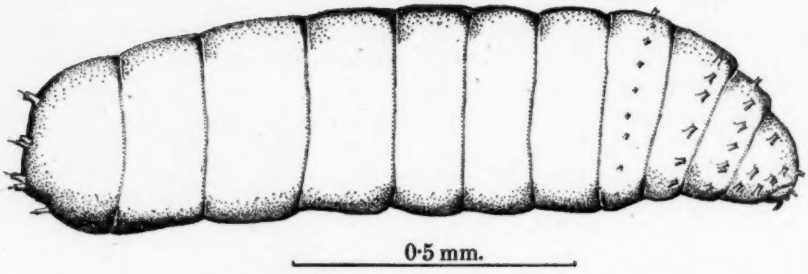

Fig. 3. Larva in 2nd instar, lateral view.

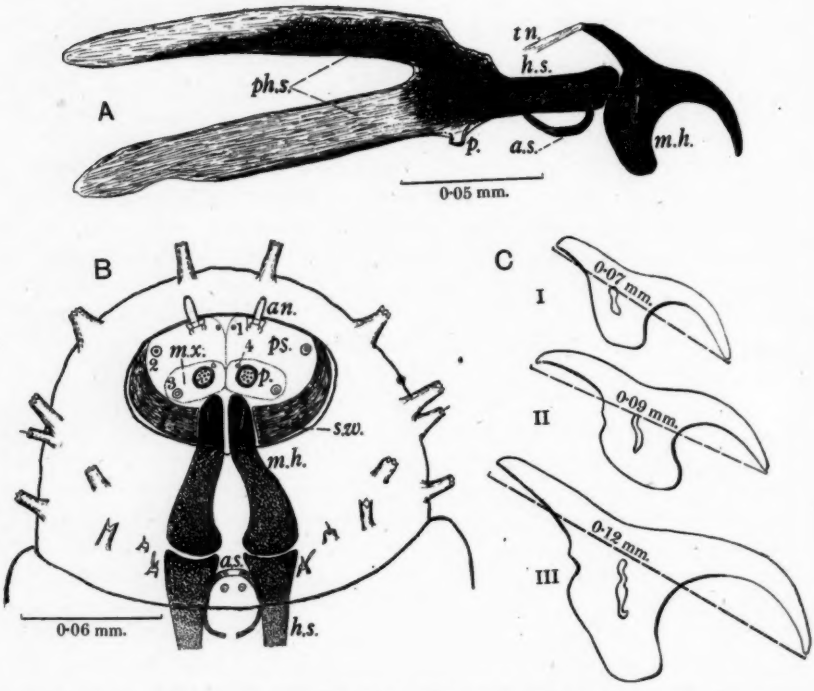

Fig. 4. A. Bucco-pharyngeal armature of 3rd instar larva, right half: *a.s.* annular sclerite (median ventral arch ?); *h.s.* hypostomal sclerite; *m.h.* mouth-hook; *ph.s.* pharyngeal sclerite and ventral process *p.*; *tn.* tendon of levator muscle of mouth-hook. B. Ventral view of pseudocephalon and 1st thoracic segment of 3rd instar larva: *an.* antenna; *a.s.* annular sclerite; *h.s.* hypostomal sclerite; *m.h.* mouth-hook; *mx.* maxilla and palp *p.*; *s.w.* sclerotized wall of pseudocephalon *ps.*; *1, 2, 3, 4* sensory papillae. C. Mouth-hooks of the three successive instars.

the 1st segment (Fig. 4) there are two such girdles. The anterior girdle is composed of four dorsal sensoria, a double dorso-lateral sensorium on either side and two latero-ventral sensoria. The posterior girdle extends over the ventral surface where its sensoria become reduced mainly to spines. On the 2nd and 3rd segments there is a single girdle to each: usually eight of the sensoria to a girdle are dorsal in position. On the 1st abdominal segment there are eight to ten dorsal sensoria and all composing the girdle are greatly reduced in size. The sensoria present on the 8th abdominal segment are less clearly disposed in a girdle than those situated on the anterior segment and, moreover, they differ markedly from them in their structure (Fig. 5). The sensoria on the anterior segments have a toothed or crenated distal margin that encloses a concave surface lined by a thin membrane which is connected with a nerve ending. Those borne on the 8th abdominal segment have a smooth distal margin that similarly encloses a concave membranous surface. From the centre of this

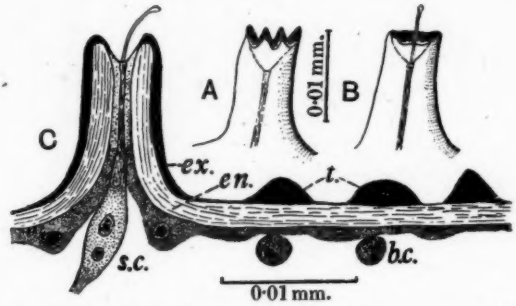

Fig. 5. Sensoria. A, from prothorax, B, from last abdominal segment and C section of the latter stained with Heidenhain's iron-alum haematoxylin: *b.c.* blood cell; *en.* endocuticula; *ex.* exocuticula; *s.c.* sensory cell; *t.* tubercles formed of exocuticula.

membrane is a spine-like process which, in some of the sensoria is slightly thickened distally. Both types of sensoria are integumentary outgrowths and, unlike sensory setae and their derivatives, they have no articular membrane. Functionally the anterior sensoria are perhaps important tactile receptors used during tunnelling. Those borne on the end segment would appear from their structure to be specially sensitive in a tactile capacity functioning perhaps when the animal progresses backwards in its burrow.

The pseudocephalon (Fig. 4) is bilobed with evident antennae (*an.*) and maxillary rudiments (*mx.*). The antennae are of the prevalent form seen among larvae of the Diptera Cyclorrhapha and the maxillary rudiments bear the usual palpi (*p*). A pair of very small sensoria is present between the antennae (*1*) and a second pair on the inner side of the maxillary rudiments (*4*). Two pairs of larger and more conspicuous sensoria (*2* and *3*) are situated ventro-laterally. The most characteristic feature of the pseudocephalon is the band of sclerotized yellowish cuticle (*s.w.*) which is probably the vestige or survival of an original sclerotized head capsule.

The bucco-pharyngeal armature is highly characteristic (Fig. 4). The mouth-hooks are stout, curved and devoid of teeth: a conspicuous feature is the large irregular foramen. Dorsally each mouth-hook is produced into a backwardly directed process which gives attachment to the tendon (*tn.*) of the levator muscle. The hypostomal sclerite (*h.s.*) is fused with the pharyngeal sclerite of its side. Situated between the two hypostomal sclerites is an annular sclerite (*an.*) which is in the form of a sclerotized hoop incomplete dorsally. It seems probable that this sclerite represents the median ventral arch or dentate sclerite of other larval Cyclorrhapha that has shifted to a more posterior position. The pharyngeal or basal sclerites (*ph.*) are devoid of longitudinal ridges and for this reason are suggestive of a carnivorous habit. A small ventral process (*p.*) is to be noted on either side. No marked differences in the bucco-pharyngeal armature are discernible in successive instars. Apart from the matter of size this armature in the 1st instar larva is much less sclerotized and amber coloured: also the process *p.* and the annular sclerite *an.* are proportionately larger than in the later instars. The relative dimensions of the mouth-hooks in successive instars are represented in Fig. 4 C.

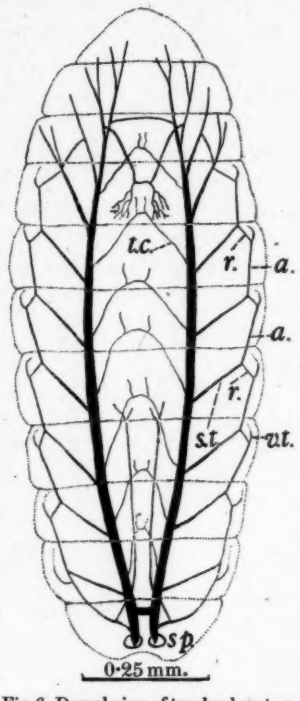

Fig. 6. Dorsal view of tracheal system of 2nd instar larva showing the main branches: *a.* anterior branch and *r.* recurrent branch of lateral segmental trachea *s.t.*; *sp.* spiracles; *t.c.* transverse commissure; *v.t.* ventral trachea.

The tracheal system (Fig. 6) is metapneustic and the chief branches are similar to those present in the larvae of most of the Diptera Cyclorrhapha. The two main tracheal trunks each give off nine lateral segmental tracheae (*st.*). These lateral tracheae bifurcate into a short recurrent branch (*r.*) and a longer anterior branch (*a.*). Where these two branches unite a ventral branch (*v.t.*) passes downwards and forwards to be distributed over the central nervous system. Dorsal transverse commissures (*t.c.*) are present in relation with the metathoracic and first seven abdominal segments: they are all tracheae of very narrow calibre. The posterior dorsal commissure situated in the eighth abdominal segment is of unusually large diameter and is extremely short owing to the approximation of the hind spiracles. Prothoracic spiracles are absent and each main tracheal trunk, instead of passing directly into the prothorax as is commonly the case in larval Cyclorrhapha, divides into smaller tracheae in the region of the metathorax.

The posterior spiracles (Fig. 7) are very small and inconspicuous. In the

3rd instar larva each spiracle is surrounded by a sclerotized peritreme and has three small ovoid openings. In the 2nd instar there are only two openings to each spiracle which is a somewhat uncommon feature. The atrophy of the anterior spiracles is perhaps to be correlated with the tunnelling habit of this larva.

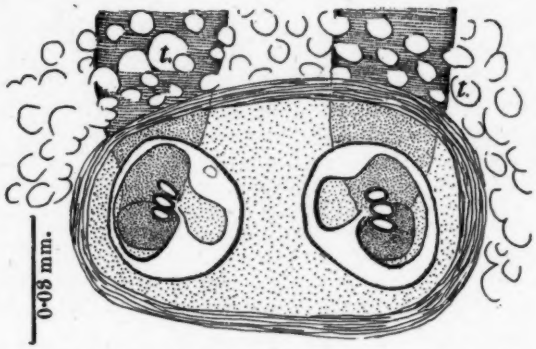

Fig. 7. Spiracles, dorsal view, of 3rd instar larva: *t.t.* cuticular tubercles.

The prepupa and pupa (Fig. 8)

The pupa of *Braula* appears to be unique among Diptera Cyclorrhapha in that it is enclosed within the unmodified cuticle of the last larval instar. This covering remains colourless, is devoid of the usual sclerotization and does not assume the typical barrel-shaped form. From the beginning of the prepupal instar up to the time of eclosion of the imago no true puparium is formed. The prepupa is cream-white and the pupa yellowish when viewed through the transparent larval skin just mentioned. The significance of the absence of the usual puparium is obscure but from analogy with the results of experiments by Fraenkel (1935) concerning puparium formation in *Calliphora*, it would appear that it may be due to cessation of a particular phase of hormone activity. By ligaturing larvae of *Calliphora* of suitable age Fraenkel showed that the region of the body thus isolated from the influence of a presumed hormone retained an unmodified cuticle. On the other hand, the cuticle covering the region subjected to hormone action became darkened in colour and heavily sclerotized in the typical manner associated with puparium formation.

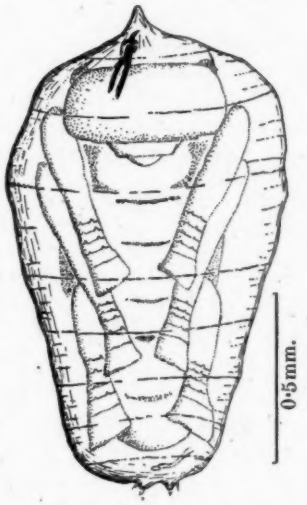

Fig. 8. Pupa of *Braula*, ventral aspect. (The specimen has contracted somewhat from the larval cuticle during preservation in alcohol.)

The transformation from the prepupa to the pupa is a gradual process and no ecdysis is undergone.

Measurements of six pupae showed that they ranged in length from 1·4 to 1·7 mm. and from 0·5 to 0·75 mm. in maximum breadth.

Affinities

Theories regarding the taxonomic position of *Braula* have been based upon characters of the imago. Its position as a member of the Diptera Pupipara remained undisputed until Börner (1908) claimed that its resemblances to *Thaumatoxena* are indicative of affinity with that genus. At first regarded by Börner as an unusual type of Hemipteron, *Thaumatoxena* was afterwards shown by Silvestri to be a member of the Phoridae. Bezzi (1916) placed *Braula* in a special subfamily of the Phoridae while Séguy (1924) and Handlirsch (1925) also regarded it as belonging to that family. Schmitz (1917) appears to be the first author to suggest that its affinities lie among the Acalypterae and considered that they are nearest to the Sphaeroceridae (Borboridae). Jobling (1936) and Hennig (1938) also regarded *Braula* as belonging to the Acalypterae. Hennig laid stress upon similarities of the male genitalia with corresponding parts in certain Drosophilidae. He further pointed out that since the Sphaeroceridae are closely allied to the Drosophilidae he was in general agreement with the conclusions of Schmitz. It may be added that Hennig's account of the family Braulidae (1938*a*) in Lindner's *Fliegen der Palaearktischen Region* is placed between the Agromyzidae (together with minor related groups), and the Chloropidae. It is noteworthy that Hendel (1937) regards the Braulidae as forming a special group—the Braulides—and places it between the Drosophilides and the Eproboscides (Pupipara).

It will be clear, therefore, that *Braula* has been referred by taxonomists to the Pupipara, to the Phoridae among the Aschiza and to the Acalypterae among Schizophora.

The inclusion of *Braula* among the Pupipara is invalidated because (*a*) it is oviparous and (*b*) its structural resemblance, in particular to the family Hippoboscidae, is due to convergence owing to its leading an epizoic, if not an actually parasitic, mode of life. The dorso-ventrally compressed body, the touch leathery cuticle, the great reduction of the eyes, the loss of both the wings and the halteres together with the massive legs armed with specialized claws are all features associated with an epizoic life and are not indicative of community of ancestry with any of the Pupipara.

The inclusion of *Braula* in the family Phoridae is also invalidated for the reason that this group is devoid of a ptilinum and, therefore, belongs to the series Aschiza. *Braula*, on the other hand, possesses a well-developed ptilinum, as was first demonstrated by Müggenburg in 1892, but this fact does not appear to have been taken into account by exponents of its Phorid relationship. Its resemblance to *Thaumatoxena* is essentially superficial and likewise due to convergence. Both insects live in the dark: they are epizoic and are subjected

to very uniform environmental conditions—in the one case in the nests of the hive bee and in the other case in the nest of termites.

The inclusion of *Braula* among the Schizophora Acalypterae is justified for reasons that are discussed at a later stage in this paper. As is well known, the last-named assemblage comprises many thousands of small and often obscure species. They are classified by different authorities into from about thirty to more than fifty separate families that are mostly based upon relatively slight differences of venation, chaetotaxy, tibial armature and so forth. This separation into so many so-called families is due more to the requirements of convenience than to scientific necessity. It will be evident, therefore, that *Braula* with its many adaptive features including the total absence of wings, together with its ill-defined chaetotaxy, displays very few diagnostic characters that would enable its position among Acalypterae to be determined. It is for this reason that attention has been given in this paper to characters displayed by the larva. Adaptive features, it may be added, are seldom so pronounced in dipterous larvae as to mask the basic structure and, for this reason, these larvae are often a valuable aid to taxonomy.

An examination of the larval characters of *Braula* leads to the conclusion that its affinities lie nearer to the Chamaemyiidae (Ochthiphilidae) than to any other family of Acalypterae. This opinion is borne out by the following characters that are common to the families Braulidae and Chamaemyiidae:

(1) The sclerotized band of cuticle around the pseudocephalon. This feature does not appear to be evident in other larvae of the Schizophora and is probably the vestige of a former more complete head capsule.

(2) The fusion of the hypostomal and pharyngeal sclerites and their general similarity of form, together with the absence of pharyngeal ridges.

(3) The simple, edentate mouth-hooks with broad bases.

(4) The general form of the antennae and maxillary palpi and the associated sensoria.

(5) The tuberculated cuticle together with segmental bands of integumentary processes.

These features, it is claimed, are indicative of mutual relationship, while there is no detailed description of any larva of the Chamaemyiidae short accounts are given by Fulmek (1912) of *Neoleucopis* Mall., by Keilin (1915) and by Pruthi & Bhatia (1938) of *Leucopis* Mg., and by Trägårdh (1931) of *Leucopomyia* Mall. The more general writings by Malloch (1921) and by Czerny (1936) were also consulted. The larva of *Leucopis* sen. str., with its elongated antennae and prominently stalked anterior and posterior spiracles, appears to be less closely related to *Braula* than that of either of the subgenera *Neoleucopis* or *Leucopomyia*. In the first-named the antennae differ but little in their relative size from those of *Braula*, the maxillary palpi appear to be very similar while the cylindrical body-form is an additional feature of resemblance. The segmentally arranged transverse bands of spinous pegs or processes ('Dornzapfen') are in all probability the forerunners of the similarly grouped girdles

of sensoria of *Braula*, which in the latter insect have become restricted to certain segments only. *Leucopomyia* resembles the larva of *Braula* more closely than either *Neoleucopis* or *Leucopis* in regard to the posterior spiracles which are sessile.

Among other features the absence of longitudinal ridges in relation to the pharyngeal sclerites is, as Keilin has clearly demonstrated, a character indicative of carnivorous habit. So far as is known the Chamaemyiidae are all predators during their larval existence and feed upon Aphidae and Coccidae. *Braula* very possibly once displayed a carnivorous habit as is suggested by the absence of pharyngeal ridges. The prominent backwardly directed process to each mouth-hook is a feature of *Braula* associated with a mining or burrowing habit. These processes consequently also tend to be developed, for example, in various Trypetidae which evidently require the increased leverage thus provided in effecting full use of the mouth-parts.

The general conclusion is that while the imagines of the Braulidae and Chamaemyiidae have undergone widely divergent evolution the similarity of structure retained by their larvae is indicative of a fundamentally close relationship.

Summary

The developmental stages of *Braula coeca* are described. The larva is an inquiline living in a tubular burrow which it makes by mining on the inner side of the capping of the honey cells in the comb of the hive bee. It is metapneustic and bears characteristic anterior and posterior girdles of sensoria.

The pupa is apparently unique among Cyclorrhapha in that it is enclosed within the unmodified cuticle of the 3rd instar larva, no puparium being formed. It is suggested that this feature is a degenerative change owing to the cessation of a particular phase of hormone activity.

The similarity of form and structure shown by the larvae of *Braula* and of the Chamaemyiidae (Ochthiphilidae) is indicative of a fundamentally close relationship. Their imagines on the other hand have undergone widely divergent evolution.

Acknowledgement is made of facilities given by Messrs L. Illingworth and C. W. Dunn for procuring material of *Braula*. Mr R. P. Bumstead, laboratory assistant, has given invaluable aid in searching numerous hives for infested combs and in removing the insect from its tunnels, while Dr W. H. Thorpe kindly provided a mounted preparation of *Leucopis bella* from California.

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SOME NEW METHODS FOR STUDYING INTESTINAL AMOEBAE AND OTHER PROTOZOA

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(With 5 Figures in the Text)

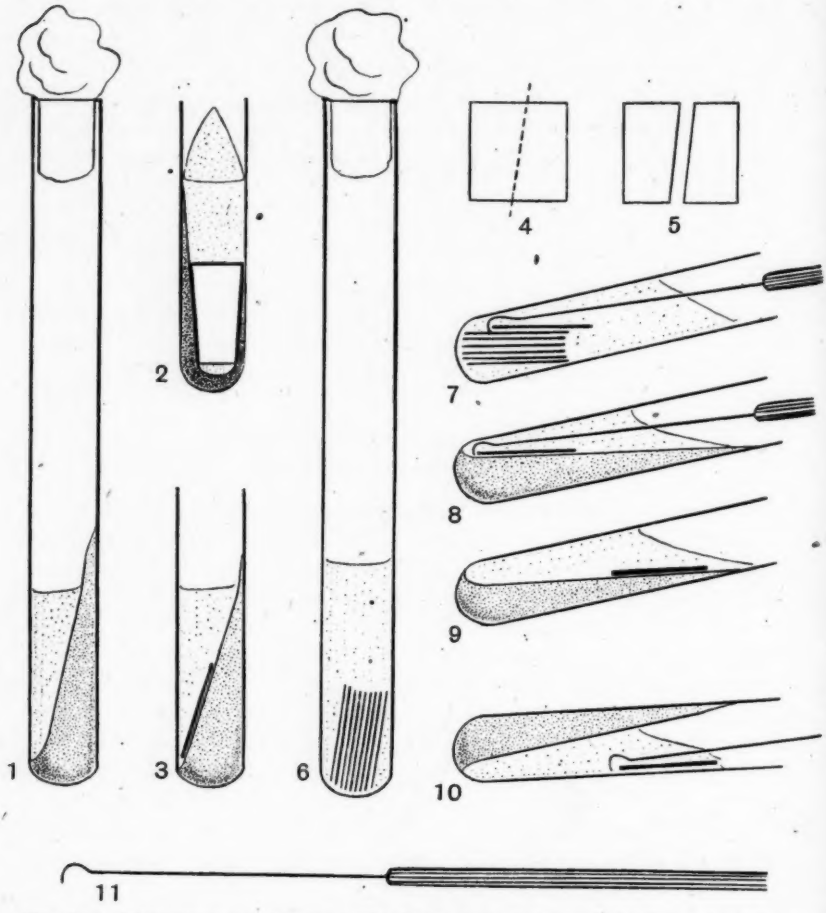
During the last few years I have successfully used a new method for investigating intestinal amoebae in cultures, and in the hope that it may also prove serviceable to other workers I now propose to describe it. At the same time I take the opportunity of noting two new staining methods which I have used especially in conjunction with this technique, but which are of general utility in protozoology and cytology.

(1) A method of obtaining permanent preparations of intestinal amoebae, from cultures, in natural attitudes

When intestinal amoebae are cultivated in the usual way, by the Boeck-Drbohlav technique, it is by no means easy to obtain satisfactory fixed and stained preparations of them at all stages of development. The amoebae lie in a mass of bacteria and other débris at the bottom of a test-tube containing a solid slope covered by liquid; and to make permanent preparations it is necessary to remove the organisms and débris with a pipette, spread them on a coverglass, and then fix the film as rapidly as possible. Good preparations can, of course, be obtained in this way, if proper precautions are taken; but even when every care is exercised—when films are spread evenly and rapidly, and all cooling, aeration, and excessive disturbance of the organisms are avoided—the results are generally disappointing. The amoebae in the final preparation are invariably found to be more or less rounded up and encrusted with bacteria, and their appearance is thus very different from that observable during life.

In order to overcome the difficulties of manipulation, and to avoid the undesirable results of the foregoing method, I have made numerous experiments during the last 17 years; and I have at last perfected a simple and effective technique which requires no particular skill and no special apparatus for its performance. By this method, anyone possessing good cultures of *E. histolytica*, or other intestinal amoebae, and the usual laboratory equipment, can easily obtain beautiful permanent specimens of the amoebae at all stages of development—in their natural attitudes, with pseudopodia extended and in every way life-like.

The method is a modification of the old one for dealing with infusions (containing free-living protozoa) by laying coverglasses on the bottom of the culture-dish or floating them on the surface-film, so that the organisms creep



Text-fig. A, illustrating the technique described in the text. 1, Diagram of culture-tube of Boeck-Drbohlav type, containing a solid slope covered by liquid—in side view: 2, 3, showing disposition of coverglass laid on the solid—in front and side view respectively: 4, method of cutting square coverglass: 5, the 2 coverglasses so cut: 6, coverglasses packed and sterilized in test-tube containing distilled water, as seen in side view: 7, method of removing top coverglass from the pack with platinum hook: 8, 9, 10, method of removing coverglass from culture by means of hook: 11, platinum hook. All figures $\frac{2}{3}$ actual size.

over the glasses and can thus be caught and fixed *in situ*. The problem has been to adapt such methods to Boeck-Drbohlav cultures, and to devise a simple procedure which can be carried out aseptically at ordinary room-

temperatures and with ordinary materials and apparatus. My method is briefly as follows:

Apparatus required. Sterile coverglasses of suitable size and shape: sterile capillary pipettes: fine forceps: a sterilizable platinum hook: and of course a good culture of amoebae, and suitable fixing and staining reagents with the necessary vessels for containing them. All operations may be conducted at ordinary laboratory temperatures.

Cultures are made and incubated in the usual way, in ordinary test-tubes about 6 in. long and ½ in. in internal diameter. These contain a solid slope (of inspissated serum or egg) covered with about 4·5 c.c. of liquid (dilute serum or egg-white). The amoebae develop at the bottom of the tube—under anaerobic conditions provided by the accompanying bacteria—at the juncture of the solid and liquid with the glass wall. The make-up of such a culture is shown diagrammatically in Text-fig. A, 1.

A platinum hook can be made by simply opening out the end of an ordinary bacteriological loop (fig. A, 11). If it is much used, however, it is better to keep a hook specially for the purpose. The wire should be not less than 2½ in. long.

The capillary pipettes, with rubber teats, are those generally used, and therefore need no particular description. (I draw mine from thin glass tubing measuring 5 mm. in external diameter.)

Coverglasses. Thin glasses (No. 0, not exceeding 0·120 mm. in thickness), ⅞ in. square, should be used. After careful cleaning, they must be cut in halves with a writing diamond. It is best to bisect them somewhat obliquely (Text-fig. A, 4), so that each furnishes two slightly wedge-shaped products (fig. 5). When a sufficient number (say 10 or 12) have been cut, they are packed together—like a pack of cards—and placed in a clean and sterile test-tube containing about 10 c.c. of distilled water. The narrower ends of the coverglasses should be towards the bottom of the tube. When this has been plugged with cotton-wool, in the usual way, the glasses can be sterilized in the autoclave, or simply by boiling,[1] and then kept for use as required. (Text-fig. A, 6 shows such a tube, with the pack of coverglasses seen edgewise.) The coverglasses must be inserted into the culture-tubes—without contaminating the cultures—and placed in the position shown in figs. 2 and 3 (2 = front view, 3 = side view): that is to say, the glass is laid on the surface of the solid at the bottom of the tube, its narrow end being directed downwards. As the solid is rounded at the bottom, and the coverglass cut square, a small semilunar space is left between the lower edge of the glass and the end of the slope. This is important.

To insert a coverglass in a culture-tube proceed as follows: Take the culture-tube and that containing the coverglasses in the left hand (as though to inoculate a subculture) and remove their plugs aseptically, in the usual way: then with the sterilized platinum hook (held in the right hand) slide off the top coverglass from the pack (fig. 7) and draw it to the mouth of the inclined

[1] Sterilization of thin coverglasses by dry heat or flaming is apt to warp them or damage their surfaces.

tube. Here it can be easily seized with fine forceps (sterilized in the flame) and transferred to the mouth of the culture-tube. Tilt this tube, so as to let the liquid overflow the inserted coverglass, and then let the latter fall to the bottom. It can be made to fit there snugly by gentle pressure with the hook.

Inoculation of tubes. Coverglasses may be laid down in sterile tubes of media—as just described—or they may be inserted (aseptically) in growing cultures of any required age. In the first case, the inoculum must be deposited —with a sterile capillary pipette—at the bottom of the tube and on the upper surface of the coverglass: in the second, the sediment at the bottom of the developing culture should be sucked into the pipette and then redistributed in the same way. (Care must be taken not to aerate the liquid by blowing bubbles into it from the pipette.) The tubes must he held aslant, of course, during these procedures (to prevent the inoculum or growing organisms from all falling to the bottom): and they are then set to incubate (or re-incubate) in the same position. They must not be placed vertically in the incubator, but with their inserted coverglasses approximately horizontal. (The flat cardboard boxes in which microscopical glass slides are sold, form, when packed with a little cotton-wool, convenient receptacles for the tubes in the incubator.)

When a culture-tube containing amoebae is thus incubated, the organisms creep over the coverglass and continue their development. They spread themselves out on the glass; feed on the accompanying bacteria, starch-grains, etc.; divide, encyst, and perform all their other actions normally: and yet they remain so firmly adherent to the glass, by their own slime, that they may readily be removed from the culture with the coverglass and fixed without becoming detached or altering their positions or shapes.

The time of incubation, in any particular case, will depend of course upon the material inoculated and the stage of development which one desires to obtain. (The necessary data can be determined by previous experiment with ordinary cultures, but are already well known for many intestinal amoebae.)

To remove a coverglass from an incubated culture-tube, and fix the amoebae crawling over it—keep the tube inclined, remove its plug, and insert the sterilized hook till its tip catches the lower edge of the coverglass (fig. A, 8). Slide the glass up the slope, by pulling the hook, until it lies near the top (fig. A, 9). Now rotate the tube till the slope lies uppermost, and with the flat of the hook detach the coverglass and let it fall to the opposite glass wall (fig. A, 10). Tilt the tube so that the liquid runs up it—but not out of it—and with the hook pull the coverglass to the top. (Be careful to keep the film on its lower surface—*i.e.* originally *upper*, where the amoebae are—covered with liquid.) When the coverglass is at the mouth of the tube it can be easily seized with the forceps, removed, and dropped (film-side downwards) into the fixative (in a hollow-ground glass block or watchglass).

All these manipulations should be carried out as quickly as possible, so as not to cool the amoebae or allow the film to be exposed to the air for more than a few seconds. They are all very easy to perform rapidly—and without

contaminating the culture—with a little practice. The technique is not so complicated as it may appear from my somewhat detailed description. It can be far more quickly performed than described.

A film so obtained can be further treated (stained, mounted, etc.) in the ordinary ways. Most of the amoebae will be found, as a rule, at the lower (narrower) end of the coverglass; only the upper (broader) end should therefore be handled with the forceps during subsequent manipulations. Some amoebae —and of course bacteria—are usually to be found on the under surface of the coverglass (where they have penetrated between it and the solid slope). These can easily be removed by wiping after fixation—at any convenient stage. (I find it best to clean the backs of the coverglasses immediately after fixation, to facilitate examination during staining.)

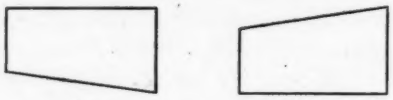
In certain cultures it may be found that, if the coverglass is left in position for a considerable time, the amoebae wander over both surfaces of the glass—or, more rarely, over the lower surface (against the solid slope) especially. It is easy to obtain permanent preparations of *both* surfaces—if required—by laying down *two* coverglasses, superposed, instead of only one. (They adhere together tightly, by capillary attraction, when removed from the stock tube, and the space between them is so small that few organisms can enter it.) When the twin glasses are removed from the culture, they can be plunged into the fixative—like a single glass—and are readily separable afterwards. Two films, back to back, are thus obtained—one glass having all the organisms on its upper surface, the other on its lower.

Films obtained in the manner described above contain—when properly made—an abundance of amoebae, permanently fixed in their natural attitudes[1] with pseudopodia extended, but relatively few bacteria. Unless the coverglass has been left in position for a long time, the bacterial film upon it is sparse, and the amoebae thus show up to great advantage. It is even possible to obtain films almost free from bacteria, by careful washing after (or during) fixation: for the amoebae adhere to the glass far more firmly than the accompanying bacteria, and these are therefore more readily dislodged.

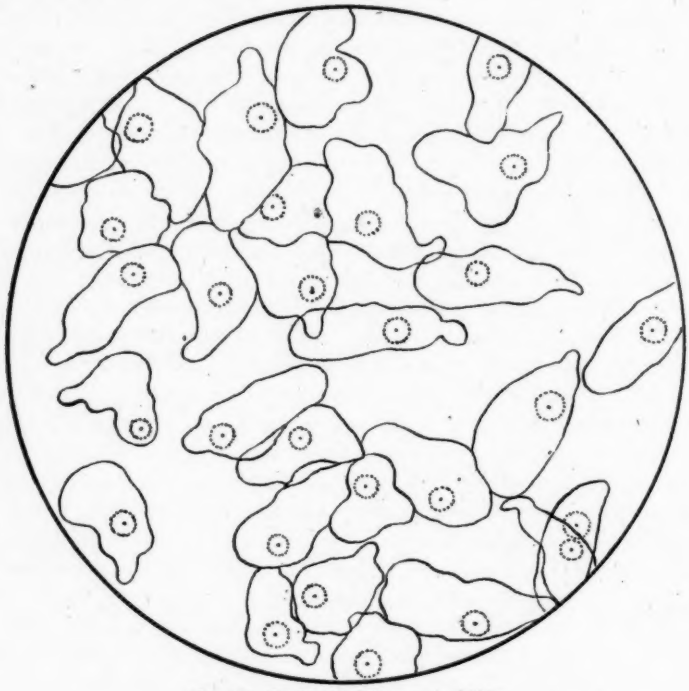
Washing the film is not necessary, but so greatly improves the appearance of the amoebae that I now never omit this step. I do it as follows: After dropping the coverglass (film-side downwards) into the fixing solution (in a hollow-ground glass block), I leave it for several minutes (usually 5–10, but longer if required). I then turn the coverglass over, so that the film lies uppermost; and then, with a pipette, squirt the fixative all over it repeatedly. This dislodges a large number of bacteria, but leaves the adherent amoebae in position. When the film looks sufficiently clean (control under microscope), it is transferred to fresh solution and left there till fixation is complete.

[1] In an earlier paper, dealing with *Dientamoeba fragilis* (see *Parasitology*, 1940, **32**, 417), I noted (p. 431) the extreme difficulty of obtaining permanent preparations of the final stages of division of this organism by the usual methods. By the methods here described I have now obtained these with ease, and have readily confirmed my previous findings.

Films prepared on coverglasses in this manner are often so thin (consisting of a single layer of amoebae and bacteria only), that they may easily be reversed inadvertently in subsequent manipulations, and thus be mounted finally with the wrong (film) side upwards—and so be ruined. This disaster may easily be avoided by the following simple precaution: It will be evident that coverglasses, cut in the manner described (see Text-fig. A, 4, 5), are



Text-fig. B. Actual size.



Text-fig. C. *E. histolytica.* ×*ca.* 1000.

asymmetrical. Of their longer sides one is square cut, the other oblique. The film may be formed on either surface, so that the coverglass, when dropped film-side downwards in the fixative, appears shaped like either *a* or *b* in Text-fig. B. If a note is made of this at the time—*i.e.* whether the glass is an *a* or a *b*—its correct orientation can always be determined at any stage afterwards without examination under the microscope.

But I need say no more, as many other details, modifications, and possibilities will readily suggest themselves to anyone who tries the method. It will be obvious, I think, that it is particularly adapted to the Feulgen "nucleal reaction", as the amoebae adhere to the coverglass so firmly that they are not detached by the somewhat drastic "hydrolysis" at 60° C. demanded by this technique. It will also be obvious that films prepared in the manner described are most suitable for photographic purposes, as the amoebae are well extended and flattened, and mostly lie in one plane.

Text-fig. C is inserted here to give a rough idea of the appearance of a good film of amoebae made by the method just described. It shows all the organisms —outlined with the *camera lucida* at a magnification of approximately 1000 diameters—in a small but typical field of a permanent preparation made from a culture of *Entamoeba histolytica* (Strain *K.* 28*c.*,[1] in Ehs+S medium). The culture was incubated at 37° C., with coverglass in position, for 17½ hours; and the film then fixed—as described—in Zenker's fluid (modified), and stained with molybdic haematoxylin.[2] Though no details—except the nuclei, in outline—are here shown, the natural shapes of the amoebae, and their abundance in the preparation, will, I think, be evident.

Two further illustrations, of other species, will be found at the end of this paper (pp. 111, 112).

(2) TWO NEW AND SIMPLE STAINING METHODS, FOR AMOEBAE AND OTHER PROTOZOA

The following methods are not entirely new, being rather modifications or applications of principles already well known. They are essentially similar to the familiar "iron-haematoxylin" methods (of M. Heidenhain and others), but involve the use of tungsten or molybdenum, in place of iron, as a mordanting agent. Salts of these metals were, of course, introduced for similar purposes (in histology) by Mallory half a century ago.

The procedure is similar, in both my methods, and consists in mordanting films (fixed by any of the usual cytological reagents) in suitable solutions of the metallic compounds, and then staining them in a ripened aqueous solution of haematoxylin (*i.e.* haematein) for an appropriate time. The great advantages of these methods—as compared, for example, with the similar iron-haematoxylin technique—are (1) their rapidity and ease, and (2) the circumstance that no differentiation under the microscope is needed after staining. Both methods are progressive.

Although, as already noted, I have used these methods particularly in conjunction with the coverglass technique outlined above, they are of general utility. They may be used equally successfully with faecal smears or with sections, and not merely for protozoa but for general cytological purposes.

[1] Described in earlier papers: see especially *Parasitology*, 1928, **20**, 365.

[2] *Vide* p. 109 *infra*.

So far as my limited experience extends, both stains are durable—provided good reagents are employed.

I call the stains—for short—"tungstic haematoxylin" and "molybdic haematoxylin". Their description follows.

(i) *Tungstic haematoxylin*

The mordant for this method is an aqueous solution of phosphotungstic acid. After much experimentation I have found a 2 per cent solution most suitable for general work, though stronger solutions (up to 10 per cent) give good results, and the concentration may be reduced—sometimes with advantage—to 1·0, 0·5, 0·25, and even 0·1 per cent. (With the weaker solutions longer mordanting is necessary.)

The mordant recommended is therefore made up by dissolving 2 g. of phosphotungstic acid[1] in 100 c.c. of distilled water. The solution keeps indefinitely and can be used repeatedly. In time it may develop a slight lilac tint, but this does not affect its activity.

The stain employed after this mordant is an aqueous solution of haematoxylin, allowed to "ripen" (*i.e.* oxidize into haematein), and may likewise be used in various concentrations. I have found a 0·2 per cent solution the most generally serviceable, but 0·5, 0·1, 0·05, and even 0·01 per cent may yield excellent results.

To make the staining solution, therefore, dissolve 0·2 g. of good quality haematoxylin (with the aid of heat) in 100 c.c. of distilled water in a small flask. Plug with cotton-wool, and leave the cooled solution in the light to ripen for a few days—shaking it occasionally. When it is of a reddish or golden brown colour, it is ready for use. (Filter into a clean stoppered bottle.) It may be used repeatedly until it turns a blackish brown (several weeks to several months, according to the quality of the original haematoxylin).

To stain fixed films, transfer them to distilled water and then place them in the mordanting solution. They may be left in this for any convenient time—but not less than 10 minutes. Half-an-hour or an hour or several hours will do. I generally leave my films in the mordant overnight, and this gives excellent results with all material tried. (The shorter times are adequate for amoebae, but the longer are better for cysts, or flagellates.)

It may be noted, in passing, that phosphotungstic acid—being itself a "fixative" for proteins—has the additional valuable property of toughening moist film preparations. Even freshly fixed films may thus be placed in the mordanting solution—without previous hardening in alcohol—with confidence that they will not fall off or macerate.

After mordanting, wash the films *thoroughly* in distilled water—several changes—to remove all excess of acid. Then place them in the haematoxylin

[1] As this is a chemical of variable and somewhat uncertain composition, I may note that I have hitherto always used that made by Merck. It is probably a phosphatodecatungstic acid of the general formula $P_2O_5.24WO_3.nH_2O$.

solution, and leave them to stain for 10 minutes or longer. They are usually well stained in 15 to 30 minutes, but can safely be left for several hours. They do not readily overstain, and the longer times are preferable for certain objects (cysts, flagellates, etc.). Examine the films under the microscope after staining, and if they appear satisfactory—with nuclei standing out sharply—wash in distilled water and then transfer to tap-water. Leave in this until they appear purple to the naked eye (30 minutes usually suffices: the water may, with advantage, be changed 2 or 3 times). Then dehydrate with alcohol, clear in xylene, and mount in balsam or dammar, or other good medium, in the usual way.

(ii) *Molybdic haematoxylin*

This method is exactly like the foregoing, with the exception that an aqueous solution of ammonium molybdate, $(NH_4)_2MoO_4$, is used instead of phosphotungstic acid as mordant. A 2 per cent solution (2 g. in 100 c.c. distilled water) likewise gives excellent results for general purposes, but the concentration may be similarly varied. The solution keeps indefinitely, but after a time a fine white precipitate forms in it. This may be filtered off without impairing its activity as a mordant.

All my remarks about the times of mordanting and staining, concentration of the mordant and staining solution, and other details, apply equally to this method. The only difference is that the stained films, when placed in tap-water finally, should appear blue rather than purple. It may be added, also, that the best results are usually obtainable with fairly fresh solutions of "haematoxylin" when ammonium molybdate is used as mordant. (Some samples of the dye have given excellent results when just made up. Solutions more than a few weeks old are generally useless.) Older and "riper" solutions yield better preparations with phosphotungstic acid.

From the foregoing notes it will be evident, I think, that tungstic and molybdic haematoxylin are both easy and convenient stains, as compared with iron-haematoxylin. They resemble the latter in colouring chromatinic elements intensely. With tungstic haematoxylin, the nuclear chromatin is stained a deep purplish black; with molybdic haematoxylin a bluish black. The cytoplasm, with both stains, is distinctly but far less intensely coloured (lilac or bluish gray respectively), so that counter-stains are unnecessary. Both stains, moreover, penetrate most amoebic cyst-walls readily, so that cysts are usually stainable as easily as free amoebae. It may be added that the chromatoids of *E. histolytica* and *E. coli* are, as a rule, only lightly stained—by both stains—in contrast with other haematoxylin methods.

Tungstic and molybdic haematoxylin also stain most bacteria distinctly, though less intensely than nuclear chromatin. This is a great advantage when studying amoebae containing ingested micro-organisms.

Moreover, both these methods—like iron-haematoxylin, but unlike other similar methods—stain the flagella of flagellates. The flagella are not, perhaps,

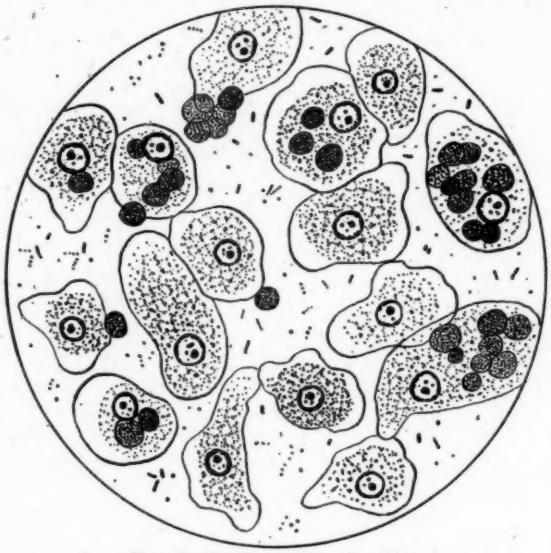
so beautifully clear as in the best Heidenhain preparations, but they can be seen easily in mounted specimens under good optical conditions. I have even obtained excellent preparations of *Trichomonas hominis*—a notoriously difficult organism to stain—with molybdic haematoxylin. In these the nucleus is sharply differentiated, the parabasal bodies and basal fibre of the undulating membrane are clearly outlined, and the flagella—with their attachments—are distinctly defined and easily countable. Ingested bacteria are also clearly visible, while the axostyle is uncoloured but distinct. All organs or inclusions, in short, are readily distinguishable. I have obtained equally good preparations of other intestinal flagellates—*Giardia*, *Enteromonas*, *Embadomonas*, etc.—with both methods of staining.

I have already noted that both tungstic and molybdic haematoxylin may be used after any of the usual fixatives. They give excellent results after Schaudinn's, Flemming's, Bouin's, or Zenker's fluid, and various other combinations of sublimate, formalin, and osmium tetroxide. Moreover—as also noted previously—they may be used to stain sections just as well as films, and this applies to vegetable as well as to animal tissues. I have found molybdic haematoxylin particularly useful for staining parasitic flagellates in sections, as it stains their flagella clearly. For example, I have easily obtained beautiful preparations of *Giardia* in sections of the cat's small intestine, and of trypanosomes (*T. rhodesiense*) in the tissues of the guinea-pig—objects not readily demonstrated in detail by other methods. I have also obtained excellent sections of coccidia (*Aggregata*, *Eimeria stiedae*, *Isospora* of the cat, etc.) and various other protozoa with both methods. Mitotic figures in the root-tip of the bean are demonstrable as clearly as in the best Heidenhain iron-haematoxylin preparations—but with less than half the time and care needed for the manipulations. Indeed I can strongly recommend both stains for all general cytological purposes.

Finally, I may note that both stains have the added advantage of colouring red blood-corpuscles electively—red with tungstic haematoxylin, bronze with molybdic. Anyone who has tried to obtain good permanent preparations of *E. histolytica* or *E. coli* containing ingested red cells will appreciate this useful though somewhat surprising property.

By the methods outlined above I have obtained excellent permanent preparations of all the intestinal amoebae of Man (*Entamoeba histolytica* and *E. coli*, *Endolimax nana*, *Iodamoeba bütschlii*, and *Dientamoeba fragilis*—all growing in cultures): but it is not possible, under present restrictions, to publish adequate pictures of my results. I therefore end these notes with two imperfect illustrations which—with the added details—may serve to show the possibilities of the methods described in previous pages. Both figures (D, E) were drawn with the aid of the *camera lucida*, and the outlines of the amoebae are therefore exact and their dimensions accurately to scale. The magnification, as here shown, is approximately 800 diameters.

Text-fig. D shows a small field from a film of *E. coli*, with the amoebae ingesting red blood-corpuscles. The preparation was made as follows: Culture-cysts of *E. coli* (Strain *MC.*), which had been kept at 2° C. for 6 months,[1] were hatched in HSre+S medium. After 5 days' incubation at 37° C. a very rich culture of amoebae was obtained. A coverglass was then laid down aseptically on the serum slope (in the manner described), and a drop of sterile human blood introduced into the culture. The amoebae and blood were carefully mixed with a pipette, and the mixture distributed over the surface of the coverglass. The culture was then re-incubated aslant. After 3 hours, the glass was removed and the film fixed in Bouin's fluid, then hardened in 70 per cent alcohol for 5 days, and stained with tungstic haematoxylin (2 per cent aqueous



Text-fig. D. *Entamoeba coli.* ×800.

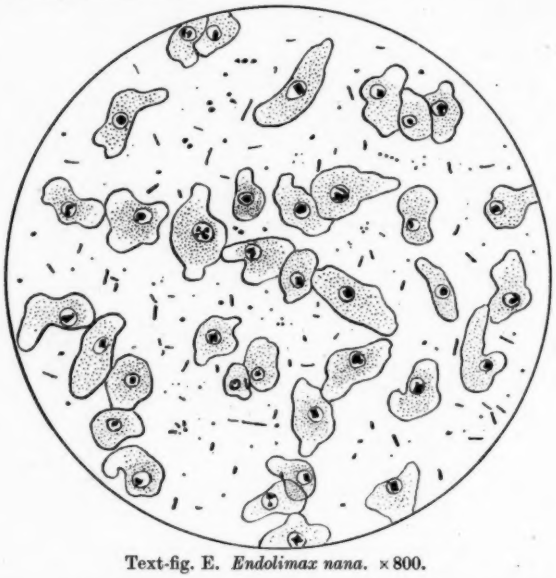
phosphotungstic acid overnight, followed by 0·2 per cent aqueous haematoxylin for about 2 hours). It was then soaked in tap-water, dehydrated with alcohol, cleared in xylene, and finally mounted in dammar. The amoebae are coloured a pinkish lilac, their nuclei an intense blackish purple, the red corpuscles a dusky red.

At first sight this film could easily be mistaken for a preparation of *E. histolytica*. But it was made from a culture obtained from typical 8-nucleate cysts of *E. coli*, and the nuclear structure of the amoebae affords a ready means of identification.

Text-fig. E shows a small field from a film of *Endolimax nana*. The strain

[1] Cysts seldom survive for so long. The culture was made in the course of experiments to determine the viability of this strain, which has been fully described in earlier papers (*Parasitology*, 1936, **28**, 541, and 1938, **30**, 195).

(*dx*) was one of many cultivated from myself after experimental infection with this species from *Macacus sinicus*.[1] The preparation was made as follows: A rich 2-day culture of amoebae in Ehs medium was inoculated into a sterile tube (of the same medium) in which a coverglass had previously been laid. The subculture was incubated aslant for 3¼ hours at 37° C., and the coverglass then removed and fixed in sublimate-alcohol (Schaudinn's fluid)+5 per cent glacial acetic acid. The film was afterwards treated with iodine, hardened in alcohol for a week, stained with molybdic haematoxylin (1 per cent ammonium molybdate overnight, 0·2 per cent haematoxylin for about 1 hour), washed, dehydrated, and mounted. The amoebae are coloured a delicate slate gray, their nuclei a deep bluish black.



Text-fig. E. *Endolimax nana*. ×800.

The characteristic forms of this species—when alive—are well shown in this preparation, and also the polymorphic structure of its karyosome: but it is not possible, in these small black-and-white figures (D and E), to depict cytoplasmic structures in detail. They are all beautifully clear in the originals.

Though the foregoing brief notes may not carry conviction, I would urge other workers to try my methods for themselves. If they do, I feel confident they will easily be able to obtain more natural and elegant permanent preparations of intestinal amoebae than anything they have ever seen before. They may also, I hope, be able to adapt these methods—as I have done—to the study of a variety of other objects.

(*MS. received for publication* 27. I. 1942.—Ed.)

[1] The experiment has already been described elsewhere (*Parasitology*, 1933, **25**, 436).

THE PENETRATION OF PYRETHRUM THROUGH THE CUTICLE OF THE TICK, *ORNITHODORUS MOUBATA* MURRAY (ARGASIDAE)

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(With 3 Figures in the Text)

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Introduction

In the life history of the tick, *Ornithodorus moubata*, the vector of human relapsing fever in Central Africa, the larva is the first stage to appear when the egg hatches. The egg membrane splits transversely usually either at the back or front, and the larva then becomes active and starts to emerge. Emergence may be complete or partial when, an hour or two after hatching, the larva ceases moving and remains thus motionless for about four days, after which the first moult takes place. The larva never feeds. When it casts its skin the first nymphal stage appears, and this and subsequent stages, including the adult, feed on blood which they suck from their host. The present experiments are concerned solely with the larva of the tick.

Methods

In the course of spraying tests with contact insecticides against the tick, it was noticed that a pyrethrum spray stimulated the immobile larvae so that they waved their legs to and fro. This fact is in keeping with the knowledge that pyrethrum stimulates, and in sufficient quantity poisons, the nervous system of insects (Hartzell & Wilcoxon, 1933; Wigglesworth, 1941). (Samples of Pyremist 'L' were kindly supplied by Messrs Stafford Allen, London. This insecticide is a solution of pyrethrins in medium shell oil P 31. The pyrethrin I content was estimated chemically, and the exact dilutions used are indicated in the tables.) It was decided to test the effect of concentration of pyrethrins on the speed of stimulation. It should be noted that pyrethrin II is probably

responsible for the stimulation (Sullivan, Haller, McGovran & Phillips, 1938), but no figures were available for the concentration of this principle. The pyrethrin I figures serve as an indication of the successive dilutions used.

The procedure was as follows: eggs were set aside in a small dish and examined daily. All hatched larvae were removed each morning, and where necessary separated from their egg shells. Thus, within 24 hr. limits the age of the larvae used was known. Except during experiments the ticks were kept in an incubator at 30° C. and approximately 50% relative humidity, and under these conditions the 72–96 hr. groups always included some larvae in the process of moulting. These were not used in the experiments.

Groups of from five to ten of the experimental larvae were dropped into a small amount of the pyrethrum-oil solution in a watch-glass, a stop-watch being simultaneously started. Only a few seconds were needed to ensure that all the larvae were completely immersed in the insecticide. The larvae were observed under a binocular microscope, and immediately each one kicked it was pushed out of the field of vision and a note made of the interval to the nearest quarter of a minute. The room temperature was observed and varied from 17 to 21° C. Since an observer's ability to follow such procedure at one sitting is limited to fairly small numbers of readings, and since in any case it is desirable to conclude the experiments as soon as possible after the larvae are collected, the latter were each day divided into groups and given the various treatments of the current experiment. In other words, the whole range of treatments in an experiment was performed on small groups each day, instead of only part of the range on all the larvae collected on a certain day. In this way it was thought that uniform variation would be obtained, even if susceptibility of the daily groups varied.

From experiments with controls dropped into pure oil (shell oil P 31, odourless distillate, etc.) it was seen that about 25% of larvae were stimulated to move within 1 min. by mere physical contact with the oil. It was therefore decided to disregard readings of 1 min. and less.

Three types of experiment were carried out: various concentrations were tested for speed of stimulation on 24–48 hr. larvae, the influence of the age of the larva on speed of stimulation was measured, and finally the effect on the speed of stimulation of different oils as carriers of the pyrethrins. The significance of the results will be dealt with in the discussion.

Concentration of pyrethrins and speed of stimulation

The results are recorded in Table 1 and Figs. 1 and 2.

It will be noticed that the coefficient of variation for the readings is rather high, the average being about 50. Contributing to this large variation in the susceptibilities of the larvae are two main causes: first, variation due to the normal distribution of susceptibility in the population, and secondly, variation due to age limits (24–48 hr.) of the larvae used, which is recorded below. A subsidiary source of variation in the speed of response was the variable room

temperature at which the experiments were carried out (17–21° C.). If the larvae had all been the same number of hours old, the coefficient of variation would have been much less. Shackell (1922) did somewhat similar experiments when he worked on paralysis and recovery times of a small marine, wood-boring Isopod, *Limnoria lignorum*, in various concentrations of phenol. For paralysis the coefficient of variation ranged from 18·39 to 29·74. In the case of the tick larvae, considering the variation due to age, the coefficients seem of the same order as in Shackell's experiments.

Owing to this large variation in the readings it is necessary to consider the significance of the difference of the means as in column 5, Table 1. Each of the values in column 5 should be at least 3 for the difference between the corresponding pairs of means to be significant. It can be seen that this condition is satisfied in the relations between 0·015 and 0·0075% pyrethrin I, and between 0·00375 and 0·0015% pyrethrin I. In addition, there is an evident trend in the figures which is seen graphically in Fig. 1 where concentration of insecticide is plotted on the abscissa and mean stimulation time as the ordinates.

Table 1. *Variation of mean time of stimulation of* Ornithodorus *larvae with various concentrations of pyrethrins*

No. of larvae	% by weight pyrethrin I	Mean time for stimulation and standard error in min.	Coefficient of variation	Significance of the difference of the means: diff./S.E. diff.
30	0·15	2·408 ± 0·192	44	
				1·25
40	0·075	2·750 ± 0·196	45	
				1·65
50	0·015	3·255 ± 0·236	52	
				3·20
50	0·0075	4·680 ± 0·382	58	
				1·90
50	0·00375	5·735 ± 0·382	48	
				3·10
50	0·0015	8·170 ± 0·679	59	

On the whole, then, the evidence presented allows the statement that the points plotted in Fig. 1 show the kind of relation existing between concentration of pyrethrin I and time of stimulation of *Ornithodorus* larvae.

So much for the statistical side of the results. The readings are also represented in Fig. 2, where the logarithm of the concentration of insecticide is plotted against the logarithm of the speed of stimulation

$$\left(\log \frac{100}{\text{time of stimulation}}\right).$$

On this logarithmic scale there is a linear relationship between concentration and speed of stimulation over the range from 0·0015 to 0·015% by weight pyrethrin I. The 0·075 and 0·15% pyrethrin I does not act with comparable speed, and the points *E* and *F* in Fig. 2 lie below the straight line *ABCD* produced. One would expect stronger insecticide to lie relatively still farther below this line. Thus from some theoretical threshold concentration (Clark, 1933), which would be somewhat tedious to ascertain, increasing strengths of pyrethrins stimulate *Ornithodorus* larvae to kicking movements with

correspondingly increasing speed, but this increase diminishes according to a logarithmic relationship (Figs. 1, 2). Finally, with concentrations above 0·015% pyrethrin I the stimulation shows increments of speed less than would be expected from this logarithmic relation (points *E* and *F*, Figs. 1, 2).

Though the interpretation must proceed with caution, certain of the bare facts may be of use in work with insecticides, as will be seen in the section on results with various media for the pyrethrins. The data from other investigations relate to the paralysing speed of the poisons (Bliss & Broadbent, 1935; Campbell, 1926; Hoskins, 1932; Shackell, 1922), whereas the basis of the present work is recordings of a much earlier stage in poisoning, viz. the initial stimulation to movement of an otherwise immobile tick. However, as outlined

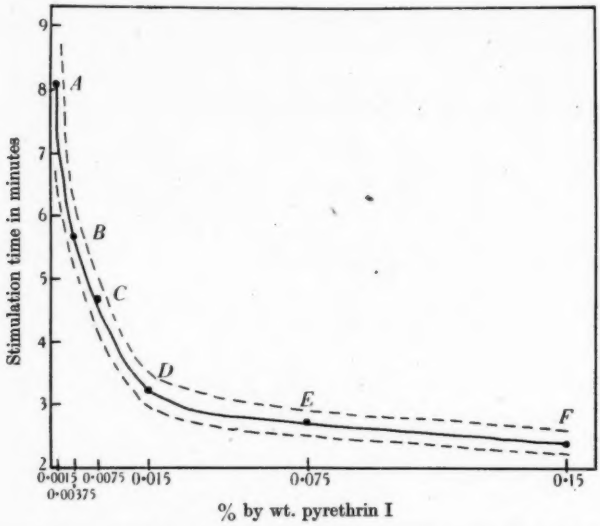

Fig. 1. Stimulation of *Ornithodorus* larvae plotted against concentration of pyrethrin I according to the data in Table 1. The broken lines follow the standard error of the mean times at each concentration.

above, the speed of stimulation varies logarithmically with concentration over a large range, and in this point agrees with the speed of paralysis as described in the papers cited.

The larva of *Ornithodorus moubata* has no spiracles or tracheal system (Dutton & Todd, 1905), and its mouth and anus are non-functional. When a larva is dipped into pyrethrum solution it seems fairly certain that the poison enters through the general integument or through particular parts of it such as the articular membranes. It then diffuses into the tissues, and, as the concentration there increases, the peripheral or central nervous system will sooner or later be stimulated to produce a kicking movement of the legs.

The difficulty is to know exactly what one is measuring. As Clark (1933)

says, 'the time measured is the time at which organisms are paralysed and hence is the sum of the delays due to chemical reactions, diffusion and lag in biological response', but again 'the most probable processes that are likely to be expressed by time are time of diffusion and this together with time for alteration of colloids in the cells'. Whereas most measurements of paralysis times include a period of quickened activity of the animal, which gradually slackens to total paralysis, in the present experiments the toxicological events in the tick occupy a minimum of time in the gross periods measured, the diffusion through cuticle and tissues a relatively large time. It seems possible that the change in slope in the velocity of stimulation curve at the point *E*, Fig. 2, may indicate when biological lag becomes a factor preponderant over concentration in deciding how soon the larva will kick.

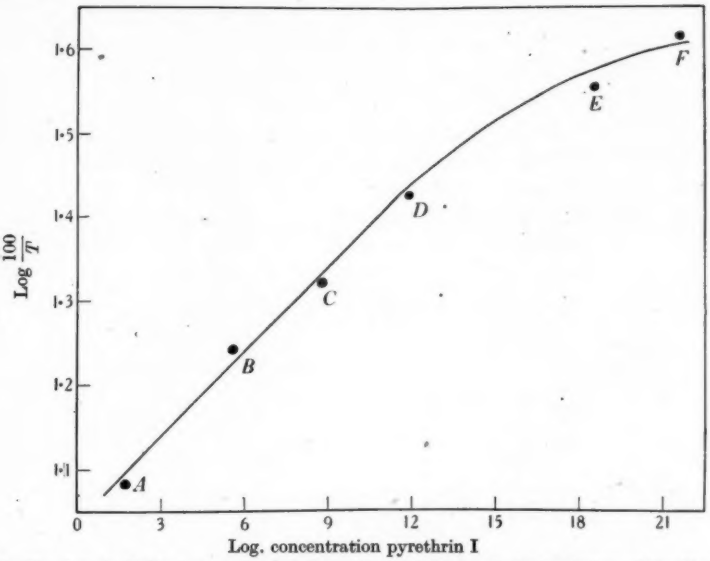

Fig. 2. The data in Table 1 plotted as the logarithm of the speed of stimulation of *Ornithodorus* larvae (log reciprocal of time × 100) against the logarithm of the concentration of pyrethrin I.

It might be possible in more detailed work to throw more light on this point in two different ways which will be mentioned in the following sections.

At the moment, then, it can be said that reaction times of the tick larvae from 8 down to 3 min. compare logarithmically with corresponding concentrations of pyrethrin I, but below 3 min. the times are longer than would be expected from this relation.

AGE OF THE LARVAE AND SPEED OF STIMULATION

Stimulation by a given concentration of pyrethrins was found to vary with the age of the larvae. At 30° C. the larval stage lasts about 4 days, so that the age of the larva being known only within 24 hr., as in the previous experiments,

four age groups were available for experiment. Groups of such age limits were found to be the most practicable and convenient to use.

The results with 0·0075% pyrethrin I can be seen in Table 2.

Table 2. *The effect of age of the larvae on speed of stimulation by pyrethrum in* Ornithodorus moubata. *The solution contained* 0·0075% *pyrethrin I by weight*

No. of larvae	Age of larvae hr.	Mean time for stimulation and standard error in min.	Coefficient of variation	Significance of the difference of the means: diff./S.E. diff.
30	0–24	1·967 ± 0·122	34	
				6·7
50	24–48	4·680 ± 0·382	58	
				3·6
30	48–72	7·275 ± 0·600	43	
				1·2
30	72–96	9·017 ± 1·386	84	

The results are shown graphically in Fig. 3, where stimulation time in minutes is plotted against age in hours. The relationship is approximately linear. From the last two columns of Table 2, it can be seen that the readings for 72–96 hr. larvae were highly variable and that the difference between these and the 48–72 hr. larvae cannot be shown to be significant. However, these 72–96 hr. larvae do differ significantly from the 24–48 hr. group (significance value = 3·1), so that the variation of susceptibility in the age may be taken as real.

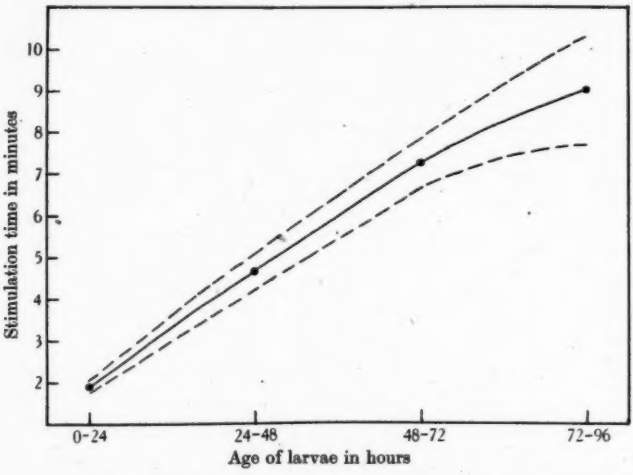

Fig. 3. Times of stimulation of *Ornithodorus* larvae by pyrethrum plotted against their age. The solution of pyrethrum contained 0·0075% by weight of pyrethrin I.

It has been mentioned above that the pyrethrins probably enter the larva through its general integument, and it has just been seen that the newly formed cuticle admits the pyrethrins more easily than older cuticle. It is probable that there is some thickening of the endocuticle after hatching and

during larval life (Wigglesworth, 1933, 1939), and this development is probably responsible for the decreased speed of entry of the pyrethrins as larval life proceeds.

There is no reason to think that the tissues of the tick become less sensitive to pyrethrum during larval life.

The large variability of the 72–96 hr. larvae (Table 2, col. 4) is due to the fact that some of them were preparing for a moult and were forming a nymphal cuticle. Those which actually showed the separation of the larval cuticle were not used for experiment.

Here may be mentioned a possible way of ascertaining whether or not the decrease in relative speed of reaction to the higher concentrations of pyrethrins (see previous section) is due to biological lag. Figs. 1 and 2 show graphs for 24–48 hr. larvae. If similar graphs were constructed for 0–24 hr. larvae, one could see in Fig. 1 whether the graphs approached a common minimum time of stimulation for the higher concentrations, and in Fig. 2 whether the graphs approached a common maximum speed of entry. If the graphs converged at the higher concentrations in spite of the differences in the cuticles of the two groups of larvae under consideration, then these conditions would point to the existence of a biological lag. Actually the 0–24 hr. larvae react in 1·967 min. (Table 2) with 0·0075% pyrethrin I, whereas with as high a concentration as 0·15% the 24–48 hr. larvae take 2·408 min. to kick (Table 1). If convergence of the graphs takes place it may be above the range of concentration here explored, and further experimentation would be necessary to elucidate this point (see last paragraph of next section).

VARIOUS MEDIA AND SPEED OF STIMULATION

The stock solution of Pyremist 'L' was a solution of pyrethrins in shell oil P 31. This solution was diluted to 10, 20, 40 and 100 times its own volume with various petroleum and vegetable oils, and the figures for the resulting pyrethrin I content were based on chemical analysis of the stock solution. The

Specifications of mineral oils

	White spirit	Odourless distillate	P 31
Specific gravity at 15° C.	0·794	0·779	0·857
Boiling point ° C.: Initial	150	198	300
Final	200	257	—
Viscosity (Redwood) sec. at 20° C.	—	32	141
Flashpoint ° C.: Open	—	—	169
Closed	—	71	160
% Unsulphonatable residue	77	99	99·2

first dilution only was used for vegetable oils, the last three only for the petroleum oils. It will thus be seen that the volume of the alternative medium varied from 90 to 99% of the volume of the mixture according to the dilution. The conclusions from the experiments are not affected by this variation. The

petroleum oils used were shell oils P 31, odourless distillate and white spirit; the vegetable oils, linseed, deodorized cotton and cold drawn castor. The results are seen in Table 3.

The most obvious thing about Table 3 is the sharp difference between the petroleum oils and the vegetable oils in their efficiency as carriers of the stimulation principle. The petroleum oils fall into a group which may be termed efficient, the vegetable oils into a group which may be termed less efficient in this respect. The methods used were the same as in the previous sections for the petroleum oils, but for the vegetable oils it was decided to stop the observations on each group of larvae after a period of time sufficient to show the difference from the petroleum group. In other words, the kicking of the more slowly reacting larvae was not awaited for longer than 15 min. To prove their healthiness they were afterwards tested for kicking in pyrethrum in petroleum oil. This procedure was economical in time and labour, and was sufficiently exact to make the above broad comparisons. Incidentally in linseed- and cotton-oil mixtures, some larvae did react in under 12 min. which represents a 'minimum mean' stimulation time. In castor-oil mixture no larvae at all reacted within 10 min., so that observation was stopped at that point.

Table 3. *The mean time of stimulation of* Ornithodorus moubata *larvae by pyrethrum in various media. Number of larvae, twenty in each case. The mean time is recorded in minutes with the standard error*

% by weight of pyrethrin I	0·044	0·022	0·011	0·0044
Oil: P 31	—	3·850 ± 0·391	3·888 ± 0·484	6·738 ± 1·225
Odourless distillate	—	2·175 ± 0·180	3·063 ± 0·354	5·350 ± 0·577
White spirit	—	2·588 ± 0·202	3·125 ± 0·363	7·307 ± 0·739
Linseed	>12	—	—	—
Cotton	>12	—	—	—
Castor	>10	—	—	—

Further comparisons within the group of petroleum oils would be interesting, but it is not thought that the present data are sufficient for this purpose. In this connexion it can be seen how useful is a knowledge of the form of the concentration-effect curve (Fig. 1) for obtaining a complete comparison of two media. With pyrethrum sprays against flies, Richardson (1932) found that distillates with the higher boiling-points were more rapid in action and more toxic than those of lower ranges.

Here may be mentioned another possible method of ascertaining whether or not the decrease in relative speed of reaction to the higher concentrations of pyrethrins is due to biological lag (see previous two sections). If two media showing significantly different stimulation times when carrying lower concentrations of pyrethrins tended to give identical stimulation times in the higher concentrations of pyrethrum, it seems likely that the limiting factor in this region is biological lag. If, on the other hand, the two media still showed a significant difference in efficiency as carriers, other explanations would have

to be sought for the decrease in relative speed of reaction to the higher concentrations. For comparisons of small stimulation times in these and other comparable experiments the method of eliminating readings of 1 min. and less would have to be examined statistically for soundness. In these experiments the number of readings of 1 min. or less was fairly consistent for all concentrations of insecticides used.

SUMMARY

1. Experiments are described in which various concentrations of pyrethrum in oil are tested for speed of stimulation of *Ornithodorus* larvae. There is a logarithmic relation between concentration and speed of stimulation, except with concentrated solutions (stronger than 0·015% by weight of pyrethrin I) in which there is a relative retardation of response.

2. As larvae grow older they respond more slowly, and this is probably due to an increase in thickness of the cuticle in natural growth.

3. Mineral oils induce much swifter penetration of the pyrethrum than any of the vegetable oils tested.[1]

My thanks are due to Prof. Buxton in whose department the work was done, and also to Dr J. T. Martin of Rothamsted Experimental Station, who kindly analysed samples of Pyremist 'L' for pyrethrin I.

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[1] Wigglesworth (unpublished work) has recently studied the entry of pyrethrins through a standard area of cuticle in the bug *Rhodnius*. He has likewise found that light oils induce more rapid penetration than heavier oils, that vegetable oils are far less effective than mineral oils and that the thickness of the endocuticle (as confirmed histologically) greatly influences the rate of penetration.

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WHIPWORMS AND BLOOD-FLUKES IN DOGS IN INDIA, WITH REMARKS ON TRICHURIASIS

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Members of the genus *Trichuris* Roederer, 1761 (Syn. *Trichocephalos* Goeze, 1782; *Trichocephalus* Schrank, 1788) have a characteristic shape. The oesophageal portion of the body is very slender and is longer than the posterior portion, the latter being usually much stouter than the former. The eggs are lemon-shaped with a knob at each end, while the egg-shell is of a dark brown colour when seen in faeces.

The genus includes several species which occur in domestic animals and one species which occurs in man. Though it has been known for a long time that whipworms occur in a number of domestic animals and even in man in India, they have not previously been reported from dogs in this country. The species found in the dog is *T. vulpis*. During the examination of material from dogs at Muktesar and other parts of India, the author observed half a dozen cases of whipworm infestation. The infestation appears to be fairly widespread in this country in view of the fact that the material examined by the author came from different and distant localities, such as Bombay, Jind State, and Bhimtal and Dehra Dun, both in the United Provinces.

The worm usually occurs in the caecum of the dog and the eggs are voided with the faeces. These develop in the open and are extremely resistant to adverse conditions. The development may take from a few weeks to several months, depending upon environmental conditions. When eggs containing infective larvae are swallowed by a suitable host, they hatch and the young worms develop for a short time in the small intestine and later migrate to the caecum. The young worms attach themselves to the mucosa of the caecum by means of a piercing lancet in the mouth. As they develop, the anterior portion of the body remains attached to the mucosa, while the posterior portion hangs free in the lumen of the caecum. They attain maturity in about three months.

The exact symptoms produced by whipworms in the dog still require investigation. Though in certain cases the worms do not appear to cause any appreciable harm, at times they are known to produce acute or chronic inflammation of the caecum. In cases of heavy infestation, elevated temperature and signs of general illness may be observed. The worm's habit of piercing the mucosa opens up small wounds which may afford entrance to secondary bacterial infection.

Though whipworms are not resistant to anthelmintics, their removal is difficult, as it is not easy to make certain that the drugs come into contact with the worms. During their passage through the stomach and intestine the drugs are considerably diluted by the contents of the digestive tract and they are also

absorbed there to a greater or less extent. Of the amount of the drug which gets as far as the ileocaecal valve, little or none will enter the caecum and reach its extremity where the worms are usually situated. Repeated doses are, therefore, probably better than a single dose. Hall found that mercurochrome given in capsules is effective in dislodging 88 % of dog whipworms. Daily administration in the morning of equal amounts of santonin and calomel, in doses of ¼–1 grain of each drug according to the size of the dog, has also been recommended. The treatment is kept up for a week, suspended for a week and then repeated for a week. More recently, higuerolatex, an active principle of *Ficus laurifolia*, has been found to give good results. In acute cases in valuable dogs resection of the caecum appears to be the only successful and sure measure for removing these parasites. Prophylaxis is purely a matter of hygiene.

Schistosoma suis Rao & Ayyar, 1933[1]

Rao & Ayyar (1935) recorded the first case of schistosomiasis in a dog from Jubbulpore. Two years later Rao reported a second case of intestinal schistosomiasis in a dog in the Madras Presidency. During the examination of material from dogs at Bhimtal (U.P.), the author found three dogs suffering from intestinal schistosomiasis. A large number of ova resembling those of *S. suis* were found to be present. This is the third report of intestinal schistosomiasis in dogs in India and the first in Northern India. A closer examination of pigs and dogs may reveal a fairly widespread occurrence of this parasite in this country.

The author is deeply grateful to Mr J. R. Haddow, I.V.S., for kindly reading the manuscript and for making valuable suggestions.

[1] According to Bhalerao (1938, 1939) *Schistosoma suis* Rao & Ayyar, 1933, and *S. incognitum*, Chandler, 1926, are identical species. The present author does not wish to deal with the question of the synonymy of these two species in this brief note, but regards the question of the identity of these species as still unsettled.

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NEW HEMIURIDS (TREMATODA) FROM INDIAN MARINE FOOD FISHES

PART III. TWO NEW PARASITES OF THE GENUS *LECITHOCLADIUM* LÜHE, 1901 (SUBFAMILY DINURINAE LOOSS, 1907)

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(With 2 Figures in the Text)

The genus *Lecithocladium* was erected by Lühe in 1901, with *L. excisum* (Rud. 1819) as the type species. In addition to the type species, the genus contains *L. excisiforme* Cohn, 1902, *L. gulosum* Linton, 1910, *L. longicaudum* Shen, 1934, *L. psenopsis*, *L. magnacetabulum* and *L. pagrosomi* Yamaguti, 1934, and *L. johnii* Yamaguti, 1938. *L. barbatum* Cohn, 1902, has been transferred to the genus *Dinurus* Looss, 1907, and *L. crenatum* (Molin, 1859) is regarded by Looss as a 'species inquirenda', while Lühe maintains that it is synonymous with *L. excisum*. The author (1937) has added two more species from Indian hosts to the genus. These are more fully described in the present paper.

Lecithocladium harpodontis Srivastava, 1937, *emend.*
(*L. harpodoni* Srivastava, 1937)

Host. *Chrysophrys datnia* Ham.
Habitat. Stomach.
Locality. Puri, Bay of Bengal.

Only two specimens of this species were collected from the stomach of one out of six specimens of the host examined at Puri in July 1935. The parasite has a cylindrical, muscular body of a deep brown colour. There are faint denticulations all over the body. In permanent mount, the type specimen measures 5·26 mm. in length and 0·7 mm. in maximum breadth, with a narrow tail 1·54 mm. long. The oral sucker is anteriorly directed and has a fringed ventral margin. It measures 0·34 × 0·44 mm. and opens posteriorly into a well-developed pharynx measuring 0·32 × 0·16 mm. An oesophagus is absent. The intestinal caeca have prominent shoulders and extend laterally to the hinder end of the tail where they terminate blindly. The acetabulum measures 0·3 × 0·4 mm. and is situated a little behind the first quarter of the body.

The two testes are small, spherical bodies lying in contact, one behind the other at the end of the first third of the body length. The anterior testis, 0·16 × 0·24 mm. in size, is slightly smaller than the posterior testis, which measures 0·18 × 0·26 mm. The vesicula seminalis is a bulb-shaped structure measuring 0·24 × 0·16 mm., and lies on a level with the testes. It is continued anteriorly into a sinuous, tubular pars prostatica, surrounded by prostate gland cells.

Terminally the pars prostatica joins the metraterm to form a long, tubular, coiled hermaphroditic duct, which is enclosed in a long, tubular hermaphroditic pouch. The genital pore is situated at the antero-ventral margin of the oral sucker, slightly to the left of the median line.

The ovary is a small, transversely ovoid structure which measures 0·24 × 0·28 mm. It is situated at a distance of 0·18 mm. behind the posterior testis. A small, semicircular shell-gland complex lies posteriorly to the ovary. Laurer's canal is present. In the absence of a receptaculum seminis, the initial part of the uterus is filled with sperms and serves as a receptaculum seminis uterinum. The vitellaria consist of eight narrow, coiled tubes. Posteriorly, the vitelline tubes do not extend very far behind the ovary, while anteriorly they scarcely extend in front of it. The uterus consists of descending and ascending coils which extend posteriorly for a short distance into the tail. It contains numerous operculate eggs, measuring 0·019 × 0·01 mm. The excretory bladder is Y-shaped, with the main stem bifurcating into two lateral cornua which unite dorsally to the oral sucker.

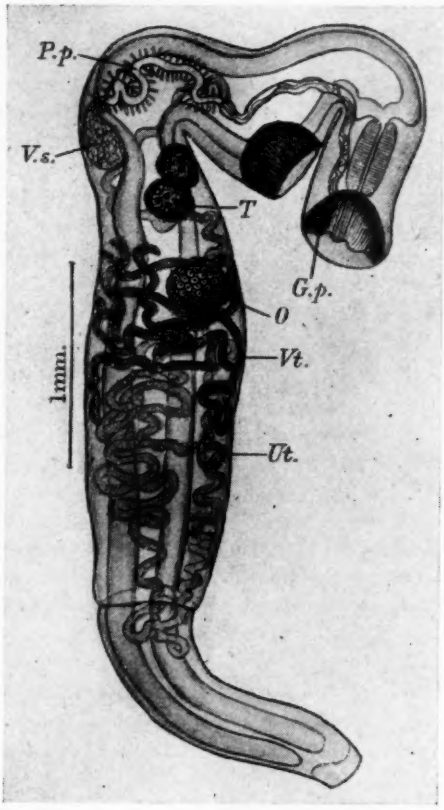

Fig. 1. Ventral view of *Lecithocladium harpodontis*. *G.p.* genital pore; *O.* ovary; *P.p.* pars prostatica; *T.* testis; *Ut.* uterus; *Vt.* vitellaria; *V.s.* vesicula seminalis.

L. harpodontis stands nearer to *L. excisiforme* in its anatomical features than to any other member of the genus. It resembles the latter in the shape and size of the body, the size ratio of the suckers, the character of the digestive system and the vitellaria, and the position of the gonads. It can, however, be distinguished from *L. excisiforme* by the position of the acetabulum, by the thin-walled vesicula seminalis and by the posterior extent of the uterus.

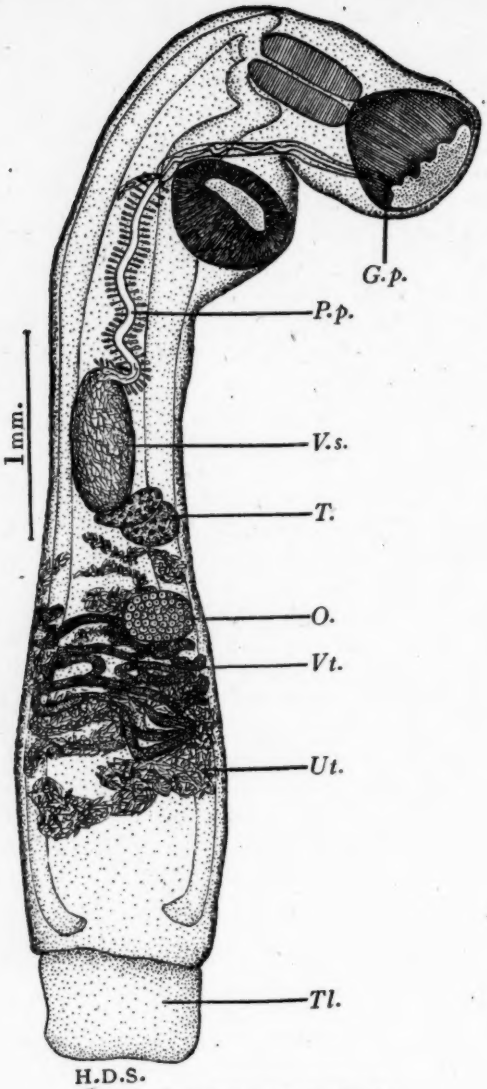

Fig. 2. Ventral view of *L. brevicaudum*.
Lettering as in Fig. 1, with the addition of *Tl.* tail.

Lecithocladium brevicaudum Srivastava, 1937, *emend.*
(*L. brevicauda* Srivastava, 1937)

Host. Chrysophrys bifasciata Forsk.

Habitat. Stomach.

Locality. Puri, Bay of Bengal.

Only one specimen of this parasite was obtained from the stomach of

Chrysophrys bifasciata caught in the Bay of Bengal and examined at Puri in July 1935. The parasite is cylindrical and very muscular, and has a broadly truncated tail. It is deep-brown in colour and is devoid of all cuticular scales or spines. The body, in permanent mount, measures 1·02 mm. in maximum breadth and 5·74 mm. in length, which includes a tail of 0·44 × 0·54 mm. The oral sucker measures 0·6 × 0·62 mm. It is a well-developed, muscular, cup-shaped structure with a fringed ventral margin. The pharynx is elongated and oval in shape and measures 0·52 × 0·26 mm. An oesophagus is absent. The intestinal caeca possess prominent shoulders and extend laterally almost to the hinder end of the body. The acetabulum is a transversely oval, muscular, cup-shaped structure situated at about the end of the anterior third of the body. It measures 0·48 × 0·62 mm.

The testes, two in number, are small, elliptical bodies measuring 0·16–0·3–0·34 mm. They are situated in tandem, overlapping each other, a little behind the equator of the body. The vesicula seminalis is a thin-walled, elongated, oval sac measuring 0·7 × 0·3 mm. It lies in the median line, partly overlapping the anterior testis. It opens anteriorly into a long, narrow, sinuous pars prostatica which is surrounded by prostate glands. Terminally, the pars prostatica joins the metraterm at the level of the middle of the acetabulum. The ductus hermaphroditicus is a long, sinuous tube, enclosed in a sac. It opens externally at the antero-ventral margin of the oral sucker, half-way between the median line and the left body wall.

The ovary is a transversely ovoid body of 0·22 × 0·3 mm. It is situated at a distance of 0·24 mm. behind the posterior testis. The shell-gland complex lies behind the ovary. Laurer's canal and a receptaculum seminis uterinum are present. The vitellaria are composed of nine slender, coiled tubes which extend from the level of the middle of the ovary to the last quarter of the body length. The uterus is transversely coiled and lies between the testes and the middle of the postvitellarian part of the body. The eggs are numerous and operculate and measure 0·019 × 0·011 mm. The excretory bladder is similar to that of the previous species.

Lecithocladium brevicaudum differs from all the other species of the genus in the length of the caeca, in the position of the testes, in the character and size of the tail and in the posterior extent of the uterus.

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NEW ALLOCREADIIDS (TREMATODA) FROM INDIAN MARINE FOOD FISHES

PART IV. THE MORPHOLOGY AND SYSTEMATIC POSITION OF A NEW GENUS, *HORATREMA*, OF DIGENETIC TREMATODES

By HAR DAYAL SRIVASTAVA, D.Sc.
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(With 1 Figure in the Text)

In the course of an extensive parasitological examination of Indian food fishes the author recovered, amongst others, six specimens of an interesting trematode from the gut of a fish examined at Karachi in June 1936. The trematode, though obviously belonging to the family Allocreadiidae, represents a new genus, *Horatrema*, and a new subfamily, Horatrematinae. This new genus forms a connecting link between *Cymbephallus* Linton, in Manter, 1934, and *Parvacreadium* Manter, 1940, of the subfamily Allocreadiinae, and the type genus *Notoporus* of the family Notoporidae Yamaguti, 1938. It will be appreciated from the systematic discussion at the end of the paper that the taxonomic status of the family Notoporidae is untenable, and it is, therefore, reduced to the rank of a subfamily, Notoporinae, under the family Allocreadiidae. Further, in view of the presence of a cirrus-sac in *Notoporus trachuri* Yamaguti, 1938, it has been considered necessary to assign it to a new genus, *Neonotoporus*.

Genus **Horatrema** n.g.

Horatrema pristipomatis n.g. et n.sp.

Host. *Pristipoma operculare* Playfair.
Habitat. Intestine.
Locality. Karachi, Arabian Sea.

Half a dozen specimens of this trematode were collected from the intestine of a marine fish examined at Karachi in June 1936. In the living state, the specimens were yellowish-brown in colour and were all of nearly equal size. The body is roughly pear-shaped and is poorly muscular. It is studded all over with extremely minute, cuticular spines. In permanent mount, the type specimen is 1·14 mm. long and 0·48 mm. broad at the level of the ovary. The oral sucker is a shallow, elliptical, cup-shaped structure measuring 0·08 × 0·18 mm. It is anteriorly directed but, in contracted specimens, it may be retracted inside the body. The muscular, spherical acetabulum, 0·16 mm. in

diameter, is situated just behind the anterior third of the body length. The oral sucker opens posteriorly through a small prepharynx into a well-developed pharynx of 0·06 × 0·05 mm. The oesophagus is a narrow tube, 0·18 mm. long. It bifurcates into two narrow lateral caeca which terminate blindly at the posterior end of the body.

The testes are two ovoid bodies situated somewhat asymmetrically in the intercaecal space at the junction of the middle and last thirds of the body length. They are separated from each other by a narrow excretory bladder. In some specimens, the testes are slightly lobed. The anterior testis is 0·18 × 0·14 mm. and is situated on the right side, while the posterior testis is 0·2 × 0·14 mm. and lies to the left of the median line. The vesicula seminalis is a fairly long, sinuous tube, with several constrictions, extending from the level of the middle of the acetabulum nearly to the anterior level of the ovary. Anteriorly the vesicula seminalis is continued on the left of the acetabulum into a long narrow duct which arches anteriorly round the ventral sucker to pass on to the right side. Terminally the duct passes into a small and inconspicuous pars prostatica, into which open a few feebly-developed gland cells.

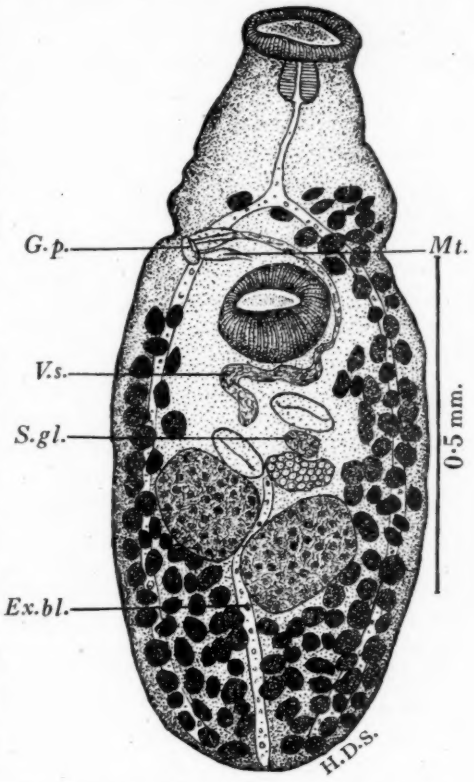

Fig. 1. Ventral view of *Horatrema pristipomatis*. *Ex.bl.* excretory bladder; *G.p.* genital pore; *Mt.* metraterm; *S.gl.* shell-gland mass; *V.s.* vesicula seminalis.

The pars prostatica and the prostate gland cells are enclosed in a small cirrus-sac. The shallow genital atrium, enclosing the male and female openings, is situated submarginally on the right side on a level with the anterior margin of the acetabulum.

The ovary measures 0·08 × 0·1 mm. and has a crenated outline. It is situated just in front of the posterior testis. A receptaculum seminis uterinum and Laurer's canal are present. The shell-gland mass lies in front of the ovary. The vitellaria consist of a large number of fair-sized follicles extending from the level of the middle of the acetabulum to the hinder end on the right side, while on the left side they continue anteriorly to the level of the intestinal

bifurcation and then extend a little on to the right half of the body. Behind the testes, the follicles of each side extend towards the median line, without meeting mesially. The uterus is confined to the intercaecal space between the acetabulum and the gonads. It contains only a few large, operculate eggs measuring 0·087–0·095 × 0·04–0·05 mm. Terminally the uterus opens into a small, oval, muscular metraterm. The excretory bladder consists of a simple, narrow tube, extending from the level of the ovary to the hinder end of the body.

In topography of gonads, development of vitellaria, position of uterus, number and size of eggs, character of excretory bladder and occurrence in a piscine host, the new genus, *Horatrema*, presents unmistakable affinities to the family Allocreadiidae Stossich, 1904. Though the general arrangement of the organs is characteristic of the family Allocreadiidae, it is evident that *Horatrema* does not fit into any of the known genera of the family. Amongst the genera of Allocreadiidae, *Horatrema* stands close to *Cymbephallus* Linton, 1934, and *Parvacreadium* Manter, 1940, both belonging to the subfamily Allocreadiinae. Both these genera have, in common with *Horatrema*, a very weak and small cirrus-sac and a receptaculum seminis uterinum. However, it differs from *Cymbephallus* notably in the position of the genital pore, the presence of cuticular spines and the configuration of the vitellaria, as well as in minor differences in the character of the oral sucker and the topography of the gonads. The American genus *Parvacreadium* can be readily distinguished from the genus here described in having its posterior end truncated and deeply indented medially into two lobes of very irregular contour. The other outstanding points of difference are the unarmed cuticle, presence of two well-developed acetabular lobes, internal thickening of the prepharynx, position of the genital pore near the pharynx, and the posterior extent of the vesicula seminalis, which does not extend beyond the acetabulum. On the basis of the differences mentioned above, it is proposed to create a new genus, *Horatrema*, and a new subfamily, Horatrematinae, under the family Allocreadiidae for the parasite described in this paper.

In 1938, Yamaguti created a new genus *Notoporus* for two new species, *N. leiognathi* and *N. trachuri*, which are parasitic in Japanese fishes. With regard to the taxonomic position of the new genus, Yamaguti remarks: 'The present genus (*Notoporus*) is characterized by the position of the genital pore and the structure of the male terminal genitalia. In these respects it differs from any of the known trematodes of fishes. A new family Notoporidae is therefore proposed for its reception and placed near the Allocreadiidae.' The genital pore in Notoporidae is situated on the dorsal side, near the left body margin, just in front of the acetabulum or level with it. The male terminal genitalia consist of a long, sinuous vesicula seminalis, extending posteriorly a short distance beyond the acetabulum, and a voluminous pars prostatica with well-developed prostate gland cells. A cirrus is absent, but a thin-walled cirrus-sac enclosing the vesicula seminalis, pars prostatica and prostate gland cells

may be present. The family Notoporidae resembles the Allocreadiidae in most of its characters, such as alimentary system, topography of gonads, configuration of uterus, development of vitellaria, character of excretory bladder, nature of vesicula seminalis, presence or absence of feebly-developed cirrus-sac and occurrence in piscine hosts. Its two distinctive features, which according to Yamaguti warrant the creation of a new family, are the location of the genital pore on the dorsal side and the presence of a voluminous pars prostatica with well-developed prostate gland cells. The location of the genital pore on the dorsal or ventral side is often difficult to determine in *in toto* mounts; and pars prostatica and prostate gland cells are fairly well developed in several genera of the family Allocreadiidae. In any case, these two features cannot be accepted as being of such taxonomic significance as to justify the formation of a new family. The Notoporidae are therefore reduced to the rank of a subfamily, Notoporinae, and assigned to the family Allocreadiidae. Under the genus *Notoporus* Yamaguti has described two species, *N. leiognathi*, the type species, and *N. trachuri*. The latter species differs from the former mainly in the presence of a well-defined, though poorly muscular, cirrus-sac and in the relative size of the suckers and the positions of the gonads and genital pore. The presence or absence of a cirrus-sac is of generic importance and hence *N. trachuri* is removed from the genus *Notoporus* and assigned as type species to a new genus, *Neonotoporus*. The diagnosis of this genus will be the same as for *Notoporus*, with the modification that a cirrus-sac is present in the former genus and absent in the latter. The subfamily Notoporinae thus comprises two genera, *Notoporus* and *Neonotoporus*. The diagnosis of the subfamily will be:

NOTOPORINAE, new subfamily

Family Allocreadiidae. Small forms with oral sucker and acetabulum well-developed, moderately wide apart from each other. Pharynx and oesophagus present. Intestinal caeca simple, long. Testes in hind-body, intercaecal. Vesicula seminalis, pars prostatica and prostate gland cells well-developed, may be enclosed in thin-walled pouch. Cirrus absent. Genital pore dorsal, in front of or on a level with the acetabulum, on left body margin. Ovary submedian and pretesticular. Uterus intercaecal, coiled between testes and acetabulum, eggs large, not numerous. Vitellaria well-developed, lateral, interrupted near genital pore. Excretory bladder tubular, not very long. Parasites of fishes. Type genus—*Notoporus*.

The genera *Horatrema*, *Notoporus* and *Neonotoporus* resemble one another in their digestive and excretory systems, in the general topography of the gonads, the arrangement of the vitellaria, the configuration of the uterus, the character and course of the vesicula seminalis and the lateral position of the genital pore. The genital pore in the Japanese genera is situated on the left side, while in the Indian genus it is on the right. This difference may possibly be attributed to amphitypy, which is of common occurrence in trematodes. The Japanese genera differ from *Horatrema* mainly in possessing

a voluminous pars prostatica with well-developed prostate gland cells and in the situation of the genital pore, which lies on the dorsal surface of the body. Another point of difference lies in the absence of spines on the cuticle. The subfamily Horatrematinae is related on the one hand to the subfamily Allocreadiinae through the genera *Cymbephallus* and *Parvacreadium*, and on the other hand to the subfamily Notoporinae. The following diagnosis will hold good for the genus *Horatrema* and the subfamily Horatrematinae.

HORATREMATINAE, new subfamily

Family Allocreadiidae. Body small, pear-shaped, spinose; oral sucker small, shallow, disk-shaped; acetabulum behind anterior third of body. Prepharynx small; pharynx well-developed; caeca long, narrow, lateral, ending blindly at hinder end. Testes two, intercaecal, compact or slightly lobed, asymmetrically placed at the junction of the last two thirds of body-length; vesicula seminalis long, tubular, sinuous, extending posteriorly almost to the ovary and continued anteriorly into a long and narrow duct which arches round the left side of the acetabulum to open into a small pars prostatica with a few prostate gland cells and enclosed in a small cirrus-sac, situated submarginally on the right side at the same level as the anterior margin of the acetabulum. Ovary slightly lobed, post-testicular, sinistral; shell gland preovarian; receptaculum seminis uterinum and Laurer's canal present. Uterus short, pretesticular; eggs few and large; metraterm small; genital atrium shallow, enclosing male and female pores. Vitellaria follicular, the follicles not equally well-developed on both sides and not meeting mesially behind the testes. On the right, the follicles extend up to the level of the middle of the acetabulum, while on the left they reach the intestinal bifurcation. Excretory bladder narrow, tubular, extending from the ovary to the hinder end. Parasitic in the intestine of marine fishes. Type genus—*Horatrema*.

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